RELATION OF VACCINAL IMMUNITY TO THE PERSISTENCE OF THE VIRUS IN RABBITS

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In a recent preliminary communication (1) we described briefly the isolation by cataphoresis of vaccine virus from rabbits entirely recovered from infection with it. By the method the virus can be concentrated from suspensions of tissues which fail to reveal infectivity in the usual tests of animal inoculation. We suggested that the immunity in virus diseases may be linked with the persistence in the body of living virus. In the present paper we intend to elaborate the subject and to offer additional experiments to support our conclusions.

The presence of virus in animals recovered from various virus infections. One of the outstanding instances of prolonged retention of a virus after recovery was that described by de Kock (2), in which the blood of a horse proved infectious seven years after an attack of equine pernicious anemia. As concerns foot-and-mouth disease (3), it was reported that a bull was infectious 2½ years after recovery (Bang), and cattle after six months (Loeffler); that the blood of a dead guinea pig contained the virus 198 days after inoculation (Fortner), and that material from the hoof of a healed animal yielded the active agent 34 days after infection (Olitsky, Traum, and Schoening). Lucas and Osgood (4) showed that the virus of poliomyelitis might be present in monkeys for five months after recovery from an acute attack of the experimental disease. They found (5) the incitant of poliomyelitis in the filtered nasal secretion of a child four months after a second attack of the disease. The first attack of the disease had occurred two years previously, and Lucas and Osgood considered the second attack a recurrence, believing that the child had harbored the virus during the interval.

The salivary gland virus of the guinea pig can be obtained at any stage after infection and throughout the subsequent period of immunity (6). A similar condition prevails in contagious epithelioma of fowls (7). And the blood of horses injected with African horsesickness virus is infectious for three months (8). Moreover, Rivers' Virus III has been found in a transplantable neoplasm of the rabbit so long as the tumor lasts. It should be noted that the host meantime developed an immunity to the virus (9).

Noguchi found vaccine virus in the lymph nodes of a rabbit 28 days after inoculation and 22 days after cutaneous immunity was established (10). Recently, Douglas, Smith, and Price recovered the virus from the ovary or testicle, from the adrenal, tongue, and brain of rabbits 41 days after infection (11). Vaccine virus, like Virus III, can persist in neoplasms of rabbits so long as these exist (12, 13). Rivers and Pearce found it after 64 days, and it had induced a resistance in the rabbit against vaccinal infection (13). Winkler (14) has reviewed the literature on the persistence of vaccinia and small pox viruses in man and cited several significant instances.

Sufficient references have now been given to indicate that the persistence of viruses in recovered, and immune, animals is not an unusual phenomenon. Special mention should be made, however, of the fact that in 1887, L. Pfeiffer concluded from clinical observation mainly that a patient recovered from vaccination, and consequently immune, harbors the virus indefinitely, immunity being lost when it disappears from the body (15).

Cataphoresis of vaccine virus. Douglas and Smith (16) have already shown that vaccine virus as contained in suspensions of tissues is, under ordinary conditions of hydrogen ion concentration, electronegative. The virus is capable of migrating in an electrical field to the positive pole at from pH = 5.5 to 8.4. In their experiments, Todd's apparatus (17) for determining the charge of bacteriophage was used.

It should be emphasized that in the present state of knowledge one cannot say definitely whether the virus itself or material containing the active agent carries the electronegative charge.

EXPERIMENTAL

Mode of Procedure

Certain steps in the mode of procedure for the isolation of small quantities of vaccine virus by cataphoresis were taken on empirical grounds. After a few preliminary experiments we discarded rheostats and substituted the resistance offered by the suspensions of tissues themselves. We employed distilled water for making the suspensions, of which about 90 to 96 cc. were used in each test, and we permitted the current to flow over a period of three hours. That the combination of these and other circumstances, to be described immediately in detail, proved satisfactory is exemplified by the fairly regular way in which virus was obtained from a number of samples that were inactive by ordinary tests.

The vaccine virus. The type of vaccine virus which was used was the neurovaccine of Levaditi (18), which we found generally active in dilutions of 1:100 million of unfiltered testicular suspensions. Rabbits were injected intracutaneously from 12 to 178 days prior to the cataphoresis test with testicular virus, and all showed, after inoculation, the characteristic vaccinal lesions in the skin. At the time these rabbits were subjected to the test for presence of virus, they were, however, wholly recovered from the infection.

The tissue suspension. Since it was desired to test the state of immunity of the rabbit after obtaining the results of cataphoresis, the skin, testicles, or spleen were removed under ether anesthesia and the animal allowed to recover.[1] The tissues were minced, then ground with sand, and made up with 100 cc. of distilled water to a homogeneous suspension. The water employed had, as a rule, a hydrogen ion concentration of 6.5 to 6.8. In some instances in which it was found desirable to increase the ionic content of the suspension, we employed as a vehicle, not water but phosphate-buffer solution (0.25 per cent KH_2PO_4) at pH = 7.5 or 7.6. The mixture was centrifuged very gently for the purpose of sedimenting large particles. The more or less turbid suspensions were used for cataphoresis, and among the several samples thus obtained, the hydrogen ion concentration varied from 6.3 to 7.8. This wide range did not influence the results of the test apparently. It should be added that the entire procedure was carried out under sterile conditions.

The cataphoresis apparatus. An adaptation was made of the method employed by Todd (17) and by Field and Teague (19). The apparatus had the following arrangement:

A lamp cord was plugged into the house current socket which carried a direct current of from 116 to 120 volts and 10 amperes. At a point on the positive wire of the cord was attached a milliampere meter with gradations on dial permitting single milliampere readings. To both positive and negative terminals zinc electrodes were attached which were cleaned at the beginning of the experiments and between times with 10 per cent sulphuric acid, and then covered with an amalgam of mercury, so as to render them non-polarizable. The electrodes were placed in a glass dish containing 10–20 cc. of supersaturated zinc sulphate solution.

On ring stands were clamped three sterilized, glass U tubes, the over-all measurements of each being 10 cm. height and 5.5 cm. width, with a uniform bore of 1.5 cm., and a capacity of from 40–44 cc. The curved part of each U tube was plugged with sterile cotton so as to prevent convection currents. The three tubes were connected with each other and with the positive and negative electrodes by means of small, glass connecting tubes, also U-shaped. The over-all measurements of the connecting tubes, with the exception of the first, the one which extended from the positive electrode to the first U tube, were 6.5 cm. height, 3 cm. width, the uniform bore being 4 mm. The first connecting tube was constructed with the electrode arm much longer, namely, 12 cm., which prevented the zinc sulphate from wandering into the material in the first U tube. The connecting tubes were filled with agar gel consisting of 1 per cent agar and 1 per cent sodium chloride in distilled water and sterilized. The pH of the agar was 6.6, but positive results could also be obtained when the reaction was adjusted to pH = 7.5 with phosphate-buffer solution or sodium hydroxide. Care was exercised to prevent inclusion of air bubbles in the tubes, which could offer gaps to the current. In addition, a volt meter with single volt readings was employed to obtain the drop in potential through the system. This varied from 115 to 119 volts in the system of U tubes,

[1] All operations on animals were made with the help of complete ether anesthesia.

or 1 to 2 volts less than the house current voltage, and was therefore between about 38 to 39 volts for each.

The test. After removal of about 5 cc. of the tissue suspension intended for cataphoresis, part of which was used to determine the hydrogen ion concentration and part the activity of the suspension, from 30–32 cc. of the remainder were poured into each of the U tubes. The connecting tubes were then adjusted so that each end penetrated the fluid in the U tube to the depth of 1 to 1.5 cm. The U tubes were plugged with sterile cotton and the glass part of the apparatus was covered with sterile cotton or gauze to prevent the entrance of air contaminants. When hydrostatic equilibrium was established, the current was turned on and allowed to run for three hours. After the first 20 minutes there appeared, as a rule, a clearing at the cathodic part of the suspension and a clouding at the anodic part of both the suspension and the agar of the connecting tubes. The change was progressive and reached its maximum after about two hours.

When the current was discontinued after three hours, the connecting tubes were removed. From 2 to 3 cm. of the agar at each end, corresponding to positive and negative poles, was scooped out with a cataract knife. Into one mortar was placed the three anodic portions, and into another the three cathodic specimens. These were ground with 1.5 to 2 cc. of Ringer's solution. The ground agar emulsion was then injected intratesticularly by the usual method into rabbits, the pooled anodic material into one animal and the cathodic into another. If the agar contained virus, characteristic vaccinal orchitis developed, first showing itself in 24 to 48 hours. In any event, after three to four days, the testicles were removed and suspensions of them were injected into the skin of normal and of vaccine-virus immune rabbits, to confirm the original diagnosis. Finally, either before or after it was learned that virus was present or absent, the animal from which the tissue was removed for the cataphoresis test was examined for its immunity. This was done by injecting endermically active vaccine virus in different dilutions of 1:10 to 1:10,000. A control normal rabbit was similarly inoculated for comparison. Immune animals showed within 24 hours the transitory Jenner-von Pirquet "immediate reaction," that is, a hyperemia at the site of the injections of the 1:10 to 1:100 dilutions, whereas the normal rabbits responded at a later period with the characteristic vaccinal lesions and subsequent secondary pustules in the case of all the dilutions.

As preliminary tests, we studied by means of cataphoresis vaccine-virus infected testicles of rabbits, at the height of reaction, suspended in phosphate buffer solution at hydrogen ion concentrations of 6, 7.2, 7.5, and 8. In thoroughly controlled experiments, at all four reactions the virus was recovered only at the positive pole. We thus confirmed the earlier observation of Douglas and Smith (16) and also controlled the mode of procedure which we adopted.

RESULTS

Presence of virus in recovered rabbits. Rabbits were examined which had been injected intracutaneously 12, 51, 56, 114, and 133 days before with neurovaccine virus. The animals had responded to the inoculation with characteristic vaccinal skin lesions. The suspensions of the testicles failed in each case to reveal vaccine virus by the ordinary test of animal inoculation; but, on the other hand, they yielded active virus after cataphoresis. The identity of the virus was revealed, first, by the characteristic dermal and testicular reactions following the injection in the manner described of pooled anodic material into normal rabbits, and secondly, by either the "immediate reaction" of immunity (the Jenner-von Pirquet phenomenon), following the injection of the same material into vaccine-virus recovered animals, or by absence of any reaction in such rabbits. The corresponding substance collected at the cathode was uniformly inactive.

It was found that the active agent could be obtained from the testicles of rabbits 133 days after inoculation and 123 days after complete recovery from cutaneous vaccine-virus infection.

Relation of persistence of virus to immunity. The following protocol illustrates the relation of immunity to the persistence or loss of virus in rabbits which had been inoculated with it a considerable time before the test for immunity was made.

Six rabbits were used in this experiment.[2] Rabbit A had been injected intracutaneously with active neurovaccine virus 133 days before the time of cataphoresis; Rabbit B, 143 days, and Rabbit C, 114 days. In the case of Rabbit D, a portion of the skin was removed for test 121 days after the cutaneous vaccination, a testicle 128 days after it, and the spleen 133 days after. The testicles of Rabbit E were taken after 171 days and the spleen after 178 days; the testicle of Rabbit F after 170 days, and the spleen after 175 days.[1] These animals had been vaccinated as follows:

Rabbit A had been injected endermically with neurovaccine virus in three sites. Into each of two areas was put 0.25 cc. of normal rabbit serum plus 0.25 cc. of virus, and into the third, 0.25 cc. of Ringer's solution and 0.25 cc. of virus. After 48 to 72 hours all the sites showed characteristic vaccinal lesions, but the individual reactions were not marked. Three other rabbits (controls for the activity of the virus) injected in a like manner with the same virus revealed similar changes in the skin.

Rabbit B had been treated as was Rabbit A and it responded in a like manner to the injections.

[2] Three of the rabbits were kindly placed at our disposal through the cooperation of Dr. Duran-Reynals of The Rockefeller Institute.

Rabbit C had been inoculated intracutaneously with a special mixture of virus plus an extract of normal rabbit testicles—a combination which, as Duran-Reynals has shown, enhances the activity of vaccine virus (20). After the endermic inoculation of 0.25 cc. of the virus and 0.5 cc. of the normal testicular extract, a typical lesion was noted which, on the fifth day, had spread considerably over the skin and was surrounded by numerous secondary pustules. The reaction was much more severe than that noted in the other five rabbits.

Rabbits D, E, and F had been injected endermically with usual stock neurovaccine testicular virus and responded to a moderate degree with dermal vaccinal lesions.

Suspensions of the organs were prepared for cataphoresis and the tests were conducted as already described. The following results were obtained:

The testicular suspension derived from Rabbit A 133 days after the endermic inoculation of virus showed after cataphoresis no activity in the testicles of normal rabbits; nor was virus found in the suspension by animal inoculation before applying the cataphoresis test. Rabbit A was then—5 days later—subjected to an immunity test. It was inoculated into the skin at various points with active testicular virus in dilutions of 1:100 to 1:6,400—to all of which the rabbit responded with characteristic primary and secondary vaccinal lesions.

A summary of cataphoresis and immunity tests on the remaining five rabbits is given in the following table.

Rabbit	Cataphoresis of suspension of	Days after endermic inoculation of virus	Virus recovered	Immunity test	
				Days after endermic inoculation of virus	Result
B	Testicles	143	No	158	Not immune
C	Testicles	114	Yes	119	Immune
D	Skin	121	No	133	Immune
	Testicles	128	No		
	Spleen	133	Yes		
E	Testicles	171	No	185	Not immune
	Spleen	178	No		
F	Testicles	170	No	183	Not immune
	Spleen	175	No		

As the protocol and the table show, four of the rabbits which had been inoculated endermically with vaccine virus and responded with characteristic vaccinal lesions failed to yield virus from their tissues

133 to 178 days later. Two rabbits inoculated similarly yielded virus, one from the testicle after 114 days, the other from the spleen after 133 days. The tissue suspensions from these latter animals were ineffective by the usual tests of animal inoculation, but active virus was obtained from them by cataphoresis. The rabbits which failed to yield virus also failed to show immunity, whereas those which retained the virus exhibited a specific resistance to virus infection.

In one instance, the virus could not be obtained from the skin on the 121st day after intracutaneous injection, nor from the testicles on the 128th day, but could be recovered from the spleen on the 133rd day. The single observation suggests that the spleen, as well as the testicle, may be concerned with the retention of virus.

Relation of persistence of virus to inherited immunity. Béclère, Chambon, and Menard (21) give an instance suggesting that vaccinal virus may be transmitted through the placenta and affect the fetus.

A patient was vaccinated during infancy and re-vaccinated on entry into the hospital, when she was eight months advanced in pregnancy. The re-vaccination caused an "immediate reaction" of hyperemia, showing that she still retained immunity. Parturition occurred one month later, when blood serum obtained from both the mother and the new-born child (umbilical cord blood) showed distinct virus-neutralizing power. In addition, two attempts at vaccination of the infant, at 6 and 14 days' age, were unsuccessful.

Ohtawara (22), in studying this problem by means of experiments on rabbits, also concludes that vaccine virus passes through the placenta and can consequently be found in the young. He believes, however, that the virus is present in the new-born only when it exists in the blood of the mother at the time of parturition.

The next experiment, while providing a parallel to this clinical instance, adds support to the view that there may be a relationship between the persistence of vaccine virus in an animal's body and immunity to it.

We injected two pregnant rabbits endermically with vaccine virus, and later, after the young were born and a few weeks' old, each of the litter was subjected to a test for the presence of virus and of immunity.

a) Rabbit A was inoculated intracutaneously with active virus 16 days before giving birth to three young. The animal responded with characteristic primary

and secondary vaccinal lesions. Two days before the birth of the young, the rabbit was normal in appearance.

Two of the young, both females, were tested 32 and 62 days respectively after birth by the injection endermically of active vaccine virus in dilutions of 1:100 to 1:1000. They proved completely resistant to infection. A male of the litter was castrated under ether when 35 days old and allowed to recover, for the purposes of a later immunity test. The testicular suspension as such proved inactive, but after cataphoresis anodic material induced in a normal rabbit characteristic vaccinal orchitis. The height of the reaction occurred on the fifth day, when the involved testicle was removed and a suspension of it injected into the skin of another normal rabbit. Dilutions of 1:10 to 1:1000 induced characteristic primary and secondary vaccinal lesions, although the same suspension in dilutions of 1:10 to 1:100 only caused the transitory "immediate reaction" after inoculation into a vaccine-immune animal. The young rabbit which had originally been castrated was injected endermically when 62 days old with active, controlled virus in dilutions of 1:100 to 1:1000. It proved completely resistant to infection.

b) Rabbit B was inoculated intracutaneously with virus 28 days before she gave birth to a litter of young. Characteristic vaccinal lesions resulted which were healed at the time of parturition. One of the young, a female, was inoculated cutaneously with virus at the age of 32 days. Lesions of vaccinal infection developed in dilutions up to 1:100,000. Two others of the litter, also females, were likewise found to be susceptible to vaccine-virus infection.

From this experiment it can be inferred that vaccine virus passes through the placenta of the rabbit and consequently can be found in the fetus. As in the case of man, the placental passage is not a constant phenomenon. The results support, therefore, the earlier observations of Ohtawara (22).

In one of the two tests just described, the virus was obtained from the testicle of one of the resistant young of a recently vaccinated mother. This young rabbit was itself resistant to vaccinal re-infection when tested more than two months after birth.

In the second test, dealing with a litter of three females from which no suitable tissues were taken for cataphoresis, all of the offspring were susceptible to vaccinal infection. In this case the mother was inoculated 28 days before the birth of the young.

DISCUSSION

The results of the experiments presented herewith would seem to suggest that recovery from vaccine-virus infection is followed by the

persistence of virus in the body for an indefinite period. During this period, the animal appears to be resistant to re-infection; on the other hand, when the virus eventually disappears from the tissues, immunity is lost. We have found that by applying cataphoresis to the tissues of animals long previously infected with vaccine virus and still resistant, we could recover the virus, whereas none could be obtained from rabbits similarly inoculated but no longer immune.

The results of recent experiments on virus-immune serum reactions are in line with the conception here discussed. The older view is that virus is destroyed by certain "virucidal" agents, or is completely eliminated from the body on recovery from infection. Recently, Todd, Andrewes, and ourselves (1, 23) have demonstrated that in some cases at least immune serum does not act either *in vivo* or *in vitro* in a manner to destroy virus. Now it has been shown as a corollary that a virus can be obtained from animals' tissues long after recovery from infection. The fact that up to the present time it has been impossible to secure active immunity against filterable-virus diseases unless living virus is used (24) can be viewed as corroboratory evidence. The unusual case of Lucas and Osgood (5) of a second attack of poliomyelitis in a child after two years can be explained as due to an absence of virus at that time in the patient, with consequent loss of immunity. The consensus of opinion is that man should be re-vaccinated within seven years because the immunity conferred by vaccination may be lost within this period. In the experiments here presented, certain rabbits failed to yield vaccine virus from their tissues after 133, 143, 170, 171, 175, and 178 days; and each of these animals was shown to be susceptible to re-infection.

CONCLUSIONS

1. By means of cataphoresis vaccine virus can be obtained from suspensions of tissue which are inactive by the usual tests of animal inoculation.

2. Active virus can be obtained by cataphoresis from the tissues of rabbits long recovered from the effects of cutaneous vaccination.

3. Evidence is brought forward which suggests that immunity in a virus disease, such as vaccinia, may be linked with the persistence in the body of living virus.

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THE EFFECT OF CATAPHORESIS ON POLIOMYELITIS VIRUS

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Recent investigations in the field of filterable viruses have been marked by a tendency to apply to the disease-producing agents of this class certain biological principles which govern the reactions of bacteria. Of particular interest have been the observations on the behavior of viruses in an electrical field (1, 2, 3). By means of cataphoresis of virus suspensions, it has been found that the effective agent could be revealed in tissues which, by the usual tests of animal inoculation, failed to show activity. This results from the migration of the virus, or possibly, of the material in a suspension carrying the virus, to one or the other pole, depending on the charge. Thus vaccine virus under ordinary conditions of hydrogen ion concentration migrates to the anode (2, 3). By this method, furthermore, vaccine virus has been isolated from rabbits wholly recovered from infection and several weeks or months after vaccination (3, 4). Finally, cataphoresis has been of use in separating vaccine virus from neutral virus-immune serum mixtures (5). Since these observations have a bearing on immunity in virus diseases, it was determined to ascertain the behavior of the poliomyelitic virus in an electrical field.

Method

The apparatus used was a modification of that described by Field and Teague (6) and by Todd (1). The details will be given by Olitsky and Long (4) in another communication. It is sufficient to say here that three large U tubes were employed, the combined capacity of which was about 96 cc. of fluid. The ends of the connecting tubes which dipped into the fluid under observation contained 1 per cent agar and 1 per cent NaCl. This furnished a convenient method for collecting the particles containing the virus, or the virus itself, which migrated with the current. From 114 to 117.5 volts and 0.8 to 4.2 milliamperes were passed for 3 hours through the fluid under examination between non-polarizable electrodes.

At the end of the period the agar plugs were removed under sterile conditions, ground in 1 cc. of Ringer's solution, and inoculated intracerebrally in monkeys.[1] The mode of preparation of the fluid will be given with each of the following experiments.

The first step was to determine whether the virus of poliomyelitis possessed the property of migrating in an electric field.

Experiment 1.—A piece of spinal cord of a monkey dead of typical experimental poliomyelitis was used. The nervous tissue was emulsified in 100 cc. of distilled water under aseptic conditions. The cataphoresis test was done as follows: milliamperes current, 0.8; potential drop, 117 volts; time, 3 hours; pH of the suspension, 6.9. After 30 minutes the fluid at the cathodes was clear and that at the anodes cloudy. After 2 hours the cathodic material was quite clear for a distance of 2 cm. from the surface and the remainder was partly clear. The agar from the positive poles contained a diffuse whitish cloud and that from the negative pole was translucent. *Macacus rhesus* 1 was inoculated intracerebrally with 1 cc. of Ringer's solution containing the ground agar from the positive poles. On the ninth day after inoculation the animal was excited and tremulous and both legs were paralyzed. On the tenth day the prostrate monkey was etherized. The gross and microscopical lesions of the spinal cord and brain were typical of poliomyelitis. *Macacus rhesus* 2 received a similar intracerebral inoculation of Ringer's solution containing the agar from the negative poles of the same experiment. On the fifteenth day after inoculation all four limbs were paralyzed and the animal was prostrate. The monkey was etherized and the gross and microscopic lesions of the nervous system were typical of poliomyelitis.

From the fact that the animal inoculated with anodic material developed poliomyelitis first, we were led to suppose that the virus probably carried a negative charge. Since the monkey inoculated with the cathodic material also contracted the disease, although after a somewhat longer incubation period, it was thought that probably the virus suspension was too strong, and part of the virus adhered to the cathodic agar. To obviate this difficulty we next employed a Berkefeld "V" filtrate of a 5 per cent suspension of the active nervous tissue.

Experiment 2.—The spinal cord from a monkey with poliomyelitis was made into a 5 per cent suspension in distilled water. The emulsion was passed through a Berkefeld "V" candle and placed in the cataphoresis apparatus. The conditions

[1] All experimental procedures on animals were made with the aid of deep ether anesthesia.

were: milliamperes, 0.8; potential drop, 116 volts; time, 3 hours. The pH of the filtrate was 6.5. Contrary to the previous experiment, in which suspension was used, the clearing in this case appeared at the positive pole and the cathodic material remained cloudy. *Macacus rhesus* 3 was inoculated intracerebrally with 1 cc. of Ringer's solution containing the ground agar from the positive pole. The fifth day after inoculation the animal was excited, weak, and ataxic. The next day a marked tremor and partial paralysis of all limbs were present. On the seventh day the animal was prostrate, and on the eleventh it was etherized. The lesions of the nervous system were typical of poliomyelitis. *Macacus rhesus* 4 (control) was inoculated the same day with the material from the negative pole. This monkey remained well throughout the period of observation.

It will be noted that the virus collected at the positive pole. It is of interest that the clouding due to migration of proteins in the fluid appeared at the pole opposite to the one at which the virus was found.

The next experiment was designed to recover by cataphoresis the virus from a non-infectious, neutral mixture of poliomyelitis virus and convalescent serum.

Experiment 3.—Poliomyelitis material of the pooled mixed virus strain which had been glycerolated for some time was made into a 5 per cent emulsion in salt solution and passed through a Berkefeld "V" filter. 50 cc. of the filtrate was mixed with an equal volume of human convalescent serum. 4 cc. of this mixture was inoculated intraspinally and intracerebrally in *Macacus rhesus* 5 (control). No symptoms developed. The material was therefore considered to have been neutralized. The remainder of the mixture was subjected to cataphoresis under the following conditions: milliamperes, 4.2; potential drop, 114 volts; time, 3 hours; pH = 6. *Macacus rhesus* 6 was inoculated intracerebrally with 1 cc. of Ringer's solution containing the agar from the positive pole. On the eleventh day after inoculation the animal was excited, slow in movement, and tremulous. Ptosis and a right facial paralysis were present. On the twelfth day the monkey was ataxic and partially paralyzed in all four limbs. The thirteenth day the prostrate animal was etherized. The gross and microscopic lesions in the spinal cord and brain confirmed the diagnosis of poliomyelitis. *Macacus rhesus* 7 (control) was inoculated intracerebrally on the same day with the emulsified agar from the negative pole of the same experiment. No symptoms developed.

The experiment shows unmistakably that active poliomyelitis virus can be separated from a non-infective, neutral mixture of the virus and its specific immune serum. We can thus conclude that the virus is not killed by the antiserum but only held in some sort of an ineffective combination. The result also confirms the previous obser-

vation that under the conditions of the experiment poliomyelitis virus bears a negative charge.

Since it was found possible to regain active poliomyelitis virus from the neutral mixture, it was thought advisable to attempt to isolate virus from an animal which had recovered from the disease.

Experiment 4.—A monkey, *Macacus rhesus* 8, was selected which had developed typical poliomyelitis 6 days after an intracerebral inoculation of virus. Almost complete paralysis of both arms and partial paralysis of the legs had existed for 4 days, followed by rapid recovery, so that by the eleventh day there was little disability. During the next 12 days the monkey ran and jumped about with almost normal vigor. At this time, or 23 days after infection, the animal was etherized and the spinal cord made into a 5 per cent suspension in salt solution. Cataphoresis was carried out under the following conditions: milliamperes, 0.8; potential drop, 117.5 volts; time, 3 hours; pH = 6.8. *Macacus rhesus* 9 was inoculated intracerebrally with the agar suspension from the positive pole. On the eighteenth day tremor, ataxia, and partial paralysis of both arms appeared. On the nineteenth the monkey was prostrate and was etherized. The gross and microscopic examination of the central nervous system confirmed the diagnosis of poliomyelitis. *Macacus rhesus* 10 (control) was inoculated intracerebrally with the material from the negative pole and 11 (control) with the suspension of central nervous system before cataphoresis had been performed. Neither animal developed poliomyelitis.

This experiment brings out the interesting fact that active virus persists in the nervous tissues for a period after recovery from poliomyelitis. Since ordinary inoculation of central nervous tissues before cataphoresis was without effect, the positive result indicates either that the antibody concentration in the tissues is sufficient to have neutralized the virus associated with it on injection in our experiment, or that in the recovered monkeys the virus is present in too minute quantity to be detected by the usual mode of inoculation. The tests described demonstrate that the virus of poliomyelitis may be separated in effective quantity from neutral mixtures of virus and immune serum or further that it can be concentrated at the positive pole from dilute virus filtrates.

CONCLUSIONS

1. Under ordinary conditions of hydrogen ion concentration the virus of poliomyelitis, as such, or associated with particles in fine sus-

pension, migrates in an electrical field to the anode. It follows that the virus bears an electronegative charge.

2. By means of cataphoresis, the virus can be recovered from a non-infective mixture of virus and specific immune serum.

3. By the same means it is possible to reveal the presence of virus in the central nervous system of a monkey which has recovered from the active stage of experimental poliomyelitis.

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ADAPTATION OF MASTITIS STREPTOCOCCI TO MILK

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It is well known that fresh milk or milk heated at 58° or 60°C. for 20 minutes will inhibit the growth of a variety of organisms, while when milk is heated at a temperature of 80°C. or more the inhibitory principle is destroyed. That different streptococci behave differently when introduced into the same milk is brought out by the following observation: The growth of the nonhemolytic mastitis streptococcus is inhibited during the first 6 or 8 hours following inoculation and then growth begins and continues rapidly; but scarlet fever streptococci implanted in portions of the same milk gradually diminish in numbers until the milk finally becomes sterile. Both organisms grow readily in milk that has been boiled for 5 minutes.

It appeared to us that a more careful study of the end of the lag phase and the beginning of growth in the case of the mastitis streptococcus might throw some light on the relation between the inhibiting agent and the implanted organisms and perhaps help to explain the mode of action of the inhibitory substance. With these points in view a series of experiments was undertaken.

Experimental

Milk was obtained principally from two cows. Care was taken to prevent contamination by drawing the milk directly from the cleansed udder into sterile bottles. It was chilled at once, freed of fat by centrifugation, heated at 58°C. for 20 minutes, and refrigerated until used. As a rule it was 2 or more days old when inoculated. The culture usually employed was the nonhemolytic mastitis streptococcus which had been kept on artificial media. Subcultures for inoculation were made in broth and used after incubation at 38°C. for 16 hours. Further details are recorded in the protocols.

The effect of milk on the growth of the mastitis streptococcus is considered in Experiment 1.

Experiment 1. Proportionate quantities of milk heated at 58°C. for 20 minutes or boiled for 5 minutes were inoculated with the diluted broth culture of the mastitis streptococcus and incubated at 38°C. in a water bath. Portions were plated with 12 cc. of melted agar (veal infusion) immediately after inoculation and at intervals thereafter, and the colonies counted after 24 hours incubation at 38°C. Previous

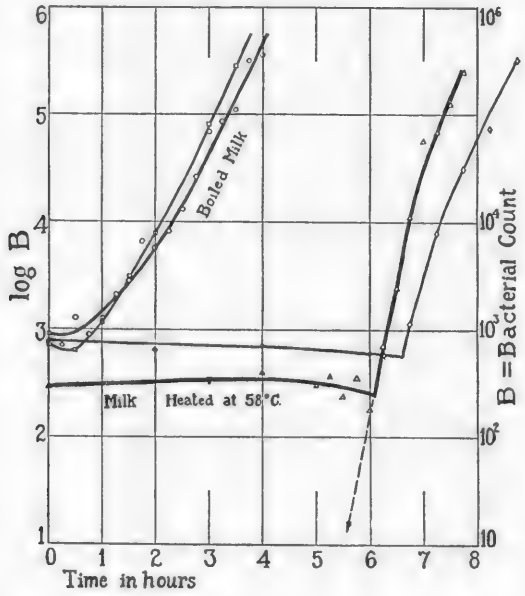

Fig. 1. Retardation of bacterial growth in milk previously heated at 58°C. for 20 minutes as compared with growth in boiled milk.

tests had shown that the lag period was relatively short in the sample which had first been boiled, so that platings were made at 15 minute intervals from the time of inoculation. The milk which had been heated at 58°C. was known to be inhibitory for this particular strain during the first 4 or 5 hours and it was only after 5 hours that frequent platings were made. The results are given in Table I and plotted in Fig. 1. They are typical for this organism under these conditions.

A similar experiment in which the intervals between platings were increased to 30 minutes showed substantially the same results, and the results have also been plotted in Fig. 1.

It will be noted from the data submitted that in the milk heated at 58°C. for 20 minutes the organisms tend to decline slightly in numbers during the first 6 hours. This phase is followed by a sharp "break" in which the organisms begin to multiply at a rapid rate and continue to do so throughout the period of observation. In the boiled milk the lag does not last longer than the first hour after which rapid growth

TABLE I

Growth of Mastitis Streptococcus in Milk

Milk boiled for 5 mins.			Milk heated at 58°C. for 20 mins.		
Time in hours after inoculation	B = bacterial count	Log B	Time in hours after inoculation	B = bacterial count	Log B
0	896	2.95	0	320	2.47
0.25	704	2.85	4.0	412	2.60
.50	1,280	3.11	5.0	307	2.48
.70	896	2.95	5.25	371	2.57
1.00	1,280	3.11	5.50	243	2.38
1.25	2,110	3.32	5.75	345	2.55
1.50	3,780	3.45	6.0	179	2.26
1.75	6,460	3.81	6.25	665	2.85
2.00	5,760	3.76	6.5	2,430	3.38
2.25	8,060	3.91	6.75	11,000	4.04
2.50	13,300	4.12	7.0	57,600	4.76
2.75	26,900	4.42	7.25	69,100	4.84
3.00	69,100	4.84	7.5	127,000	5.10
3.25	85,100	4.93	7.75	250,000	5.40
3.50	108,000	5.04	8.0		
3.75	313,000	5.50			
4.00	350,000	5.55			

begins. Repeated experiments have regularly shown that the onset of growth is sudden in milk heated at 58°C.

Three explanations to account for the sudden onset of growth in the milk previously heated at 58°C. for 20 minutes presented themselves: (1) That the inhibitory substance is utilized at some time during the lag period with the result that the uninhibited organisms grow rapidly; (2) that a resistant form is present from the start but only slowly multiplies to an appreciable number; and (3) that at some time during the lag period a resistant form develops which is capable of rapid

growth. The next three experiments were designed to test the importance of these factors.

TABLE II

Demonstration that the Inhibitory Substance Is Not Utilized during the Lag Phase

(B = bacterial count, *i.e.*, number of colonies per cc.)

A

Time in hours after inoculation	Boiled milk		Milk heated at 58°C.	
	B	log B	B	log B
0	384	2.58	358	2.55
2	4,740	3.68	409	2.61
4	86,000	4.93	499	2.70
5			1,340	3.13
5.5			1,730	3.24
5.75			2,500*	3.40*
6.0			3,700†	3.57†
6.25			9,340¶	3.97¶
6.5			26,600	4.43

B

Behavior of the Mastitis Streptococcus in Portions of A Removed at Various Times

Time in hours	5½ hr. portion,* boiled		6 hr. portion,† 58°C.		6½ hr. portion,¶ 58°C.		Control portion reheated at 58°C. after 6 hrs. incubation	
	B	log B	B	log B	B	log B	B	log B
0	576	2.76	768	2.89	640	2.81	768	2.89
1	2,300	3.36	435	2.64	512	2.71	410	2.61
2	9,120	3.96	420	2.62	576	2.76	435	2.64
3	46,100	4.66	450	2.65	380	2.58	340	2.53
4	∞	∞	250	2.40	1,540	3.19	320	2.50
5	∞	∞	2,000	3.30	32,000	4.50	310	2.49
6	∞	∞	5,760	3.76	∞	∞	510	2.71

* † ¶ The symbols indicate portions of A removed, heated and tested in B.

Experiment 2. Milk handled in the same manner as in Experiment 1 was divided into two lots. One lot, for control purposes, was boiled before inoculation and plated at 2 hour intervals during incubation at 38°C. The other lot was heated at 58°C. for 20 minutes, and likewise inoculated when cool. Portions of it were plated at 2 hour intervals for a time. The intervals were later decreased to

15 minutes. 10 cc. samples were removed after $5\frac{3}{4}$, 6, and $6\frac{1}{4}$ hours incubation. The first tube of inoculated milk, removed after $5\frac{3}{4}$ hours incubation, was boiled 5 minutes; and the other two, removed at 6 and $6\frac{1}{4}$ hours, respectively, were heated at 58°C. for 20 minutes to kill the implanted streptococci. A fourth tube of milk handled in the same manner but uninoculated was incubated for 6 hours and reheated at 58°C. It served as an additional control. All four samples were kept in the refrigerator overnight and in the morning were reinoculated with the culture and incubated at 38°C. Portions were plated each hour. The data are given in Table II and Fig. 2.

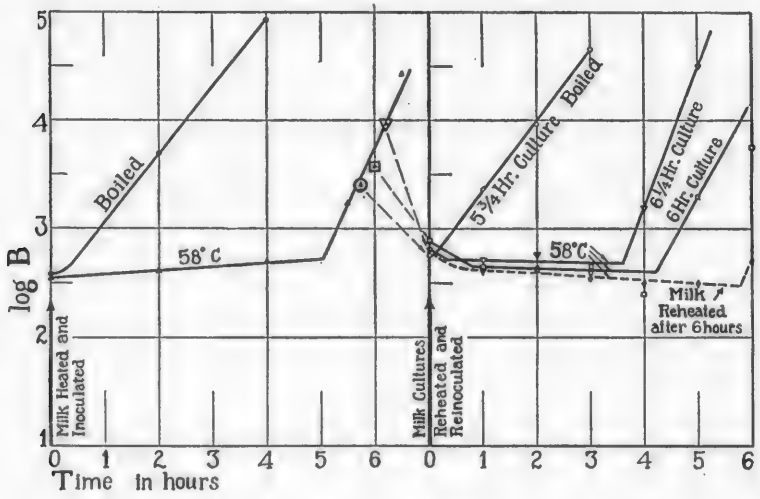

Fig. 2. Demonstration that the inhibitory agent is not utilized during the lag period.

It will be noted that the samples removed from the inoculated milk after 6 and $6\frac{1}{4}$ hours and then heated sufficiently to destroy the inoculated streptococci were still capable of producing considerable lag. It is evident that, though growth had taken place, the principle had been only slightly utilized. The contents of one of the control tubes inoculated and incubated for $5\frac{3}{4}$ hours and then boiled afforded a good culture medium from the first, whereas the action of the principle in the uninoculated milk had not been impaired either by incubation or repasteurization.

Since this experiment showed that the inhibitory substance had not been completely utilized, attention was turned to the possibility of an adaptation to the inhibitory substance on the part of the organism. The next two experiments furnished evidence compatible with the view that the organism adapts itself to the inhibitory principle.

TABLE III

A. *Effect of Fresh Milk on the Mastitis Streptococcus during the Lag Phase and the Beginning of the Growth Phase*

Time in hours	Boiled milk		58°C. milk	
	B	log B	B	log B
0	512	2.71	640	2.80
2	3,840	3.58	434	2.64
4	39,000	4.59	512	2.71
4.5	87,700	4.94	578*	2.76
5.5	495,000	5.70	1,540†	3.19
6.5	∞	∞	13,100¶	4.12
7.5	∞	∞	67,000§	4.83

B. *Subcultures Made by Centrifuging A and Exposing the Streptococci to the Action of Fresh Milk (Heated at 58°C.)*

Time in hours	4½ hr. culture*		5½ hr. culture†		6½ hr. culture¶		7½ hr. culture§	
	B	log B	B	log B	B	log B	B	log B
0	1,020	3.01	2,050	3.31	14,100	4.15	65,300	4.81
0.5	1,020	3.01	3,580	3.55	20,500	4.31	76,800	4.88
1	1,020	3.01	5,450	3.74	28,800	4.46	107,000	5.03
2	7,040	3.85	26,800	4.43	78,000	4.89	214,000	5.33
3	14,100	4.15	47,400	4.68	212,000	5.33	506,000	5.70
4	58,900	4.77	494,000	5.69	730,000	5.86	∞	∞

* † ¶ § These symbols indicate subcultures in B derived from the respective portions of A.

Experiment 3. 25 cc. of fresh milk which had been heated at 58°C. for 20 minutes, and 5 cc. of the same lot which had been boiled for 5 minutes were inoculated with proportionate quantities of mastitis streptococci. Both tubes were incubated at 38°C. and portions plated at regular intervals. 5 cc. portions of the milk that had been heated at 58°C. were removed at 4½, 5½, 6½, and 7½ hours and centrifuged. The streptococci obtained from the sediment were mixed with

5 cc. of fresh milk previously heated at 58°C. and again incubated. Care was taken not to chill the tubes during the process and the fresh milk was warmed to 38°C. before it was added. Portions of each series were plated at intervals. The results are given in Table III and Fig. 3.

It will be seen from the data that the period of inhibition extended to about the 5th hour in the milk that had been first heated at 58°C.,

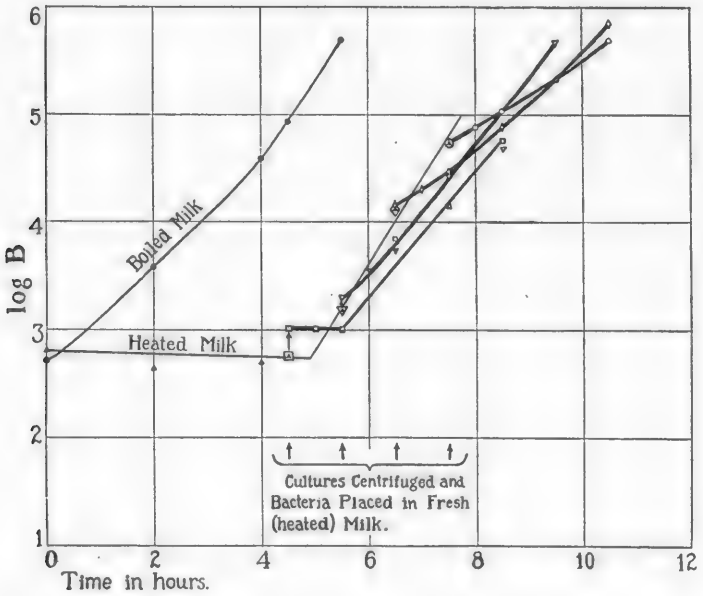

Fig. 3. Effect of fresh (pasteurized) milk on the rate of growth of mastitis streptococci removed from the original milk culture.

then the usual sharp break occurred and rapid growth ensued. The bacteria removed about one half hour before the lag phase ended failed to multiply during the first hour of exposure in fresh milk but grew rapidly thereafter. Thus in spite of the exposure to fresh milk the period of lag was about the same. On the other hand the organisms removed during the active growth phase ($5\frac{1}{2}$, $6\frac{1}{2}$, and $7\frac{1}{2}$ hours after inoculation) all grew without lag in the fresh milk.

Experiment 4 shows that the streptococcus adapts itself to milk.

Experiment 4. Milk previously heated at 58°C. for 20 minutes was distributed in sterile tubes as follows: 1 tube containing 5 cc., and 4 tubes each containing 4 cc. To the tube containing 5 cc., 0.1 cc. of a 16 hour broth culture of the mastititis streptococcus was added and a sample plated at once to determine the number of streptococci. The tube was then placed in the water bath at 38°C. At the end of 1½ hours, 1 cc. was removed and added to one of the tubes containing 4 cc. of milk. A portion of the contents of the original tube was plated as was the

TABLE IV

Total time of incubation in hours	Tube No.	Contents	Time of incubation for each tube in hours	Colonies in plates = $\frac{BD}{625}$	Log $\frac{BD}{625}$	B = calculated bacteria per cc.
A. *Adaptation of the Mastitis Streptococcus to Frequently Added Milk*						
0	1	0.1 cc. culture + 5 cc. milk	0	1,600	3.20	1,000,000
1½			1½	1,280	3.11	800,000
1½	2	1 cc. from Tube 1 + 4 cc. milk	0	1,088	3.04	136,000
3.0			1½	832	2.92	104,000
3.0	3	1 cc. from Tube 2 + 4 cc. milk	0	896	2.95	22,400
4½			1½	704	2.85	17,600
4½	4	1 cc. from Tube 3 + 4 cc. milk	0	768	2.87	3,840
6.0			1½	665	2.82	3,330
6.0	5	1 cc. from Tube 4 + 4 cc. milk	0	640	2.81	640
7½			1½	3,456	3.54	3,456
B. *Control Experiment with Boiled Milk*						
0	1	0.1 cc. culture + 5 cc. boiled milk	0	1,280	3.11	800,000
1½			1½	10,300	4.01	6,440,000
1½	2	1 cc. from Tube 1 + 4 cc. boiled milk	0	11,300	4.05	1,410,000
3.0			1½	52,400	4.72	6,550,000
3.0	3	1 cc. from Tube 2 + 4 cc. boiled milk	0	57,000	4.76	1,430,000
4½			1½	∞	∞	∞

mixture in the second tube. After incubation for another 1½ hours, 1 cc. of the second tube was added to 4 cc. of milk, and plates were prepared as before. This procedure was repeated at the end of 4½ hours and 6 hours, so that the inoculated streptococci were regularly diluted and exposed to the action of fresh milk at intervals of 1½ hours. A control series was carried on in boiled milk. The plate cultures were always prepared with the same amount of fluid.

With respect to the first tube the five tubes had dilutions of 0, 1:5, 1:25, 1:125, and 1:625 respectively. In order that the bacterial counts might be comparable the milk was diluted with sterile NaCl solution before plating, as

follows: 1:625, 1:125, 1:25, 1:5, and 0, respectively, thus making all platings on a dilution of 1:625 of the mixture in the first tube.

If B is the bacterial count per cc. and D is the dilution (1, 5, 25, etc.), then BD/625 represents the count in each plate. These values are given in Table IV.

In spite of the fact that the mastitis streptococcus was exposed to the action of fresh milk at intervals of $1\frac{1}{2}$ hours it was able to establish

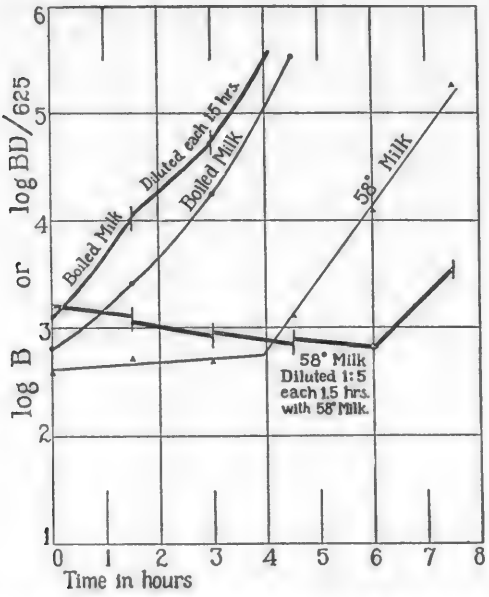

Fig. 4. Effect of milk added at frequent intervals on the growth of the mastitis streptococcus. The bacterial counts are corrected for the amount of dilution.

itself and begin to grow. The procedure, however, had two recognizable effects: first, it increased the lag period from about 4 hours in the milk control tube to about 6 hours in the experimental series, and further, there was a gradual but consistent decline in the number of streptococci until growth began. When the same experiment was done with boiled milk the streptococci increased throughout and the addition of fresh portions of boiled milk failed to retard growth.

Aside from the fact that the mastitis streptococcus becomes adapted to the inhibitory effect of milk the results in Experiment 2 indicated that a small portion of the principle had been utilized during the early phase of active growth. Experiments earlier reported[1] have shown that when milk is heavily seeded with bacteria and incubated sufficiently long to insure multiplication the inhibitory effect disappears. The question arises as to whether the utilization can be considered an adsorption by the bacterial cells. Experiment 5 is designed to throw light on this phase of the problem.

TABLE V

Bactericidal Activity of Milk Previously Treated with Living or Dead Scarlet Fever Streptococci

Time in hours	Milk heated at 58°C.		Milk incubated and refrigerated, then heated at 58°C.		Boiled milk (combined from all tubes)
	Incubated with dead streptococci, then centrifuged	Control milk	Incubated with live streptococci, centrifuged and heated at 58°C.	Control milk	
0	428	428	384	384	576
2	345	166	320	217	13,820
4	192	153	230	77	∞
6	12	4	12	2	∞
8	5	1	3	6	∞
24	0	0	0	0	
48	0	0	0	0	

Experiment 5. Mixed milk from five cows was chilled and freed from fat and 15 cc. amounts placed in four sterile tubes. Two of the tubes were heated at 58°C. for 20 minutes. One tube served as control, and to the other were added scarlet fever streptococci from 500 cc. of 48 hour broth culture which had been heated at 60°C. for 20 minutes. The two other tubes were not heated until after incubation. To one of these the scarlet fever streptococci from 500 cc. of 48 hour broth culture were added. All four tubes were incubated 2 hours and refrigerated 18 hours. The second pair of tubes, which had not been heated, were then heated at 58°C. for 20 minutes. All four tubes were centrifuged and the supernatant fluid was distributed into small tubes and reinoculated with scarlet fever streptococci. The results are given in Table V.

[1] Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1927, **45**, 319.

The experiment was also performed with large quantities of mastitis streptococci. In no case was the inhibitory substance appreciably removed by dead streptococci or by viable organisms, provided always that the tubes were not incubated sufficiently long to permit growth.

DISCUSSION

The experiments have shown that when mastitis streptococci are introduced in small numbers into milk previously heated at 58°C. for 20 minutes there is a lag in growth of 5 or 6 hours succeeded by an abrupt increase which continues at a rapid rate (Fig. 1). The experiments further indicate that the inhibitory principle is not destroyed during this period since sufficient remains to inhibit the growth of a similar culture for about 4 hours (Fig. 2). Furthermore had the substance been completely destroyed during the lag period the break in the curve would not have been as sharp.

The explanation that a resistant form is present from the start and requires a period of time to multiply to an appreciable number, can be immediately discarded from the shape of the curves in Fig. 1. Here we have plotted the logarithms of the bacterial count against time of incubation. Milk previously heated at 58°C. is shown to produce a long lag, followed by a sharp break and rapid growth. The experiment was performed enough times and at intervals sufficiently close for us to be certain of the abruptness of the change. Even if only one bacterium of a strain resistant to the inhibitory agent had been present at the time of inoculation, this one bacterium, if it multiplied at the rate shown to be typical of the surviving forms, could have produced a lag of not more than 3 hours. This calculation takes into account the normal lag of 1 hour which is found when the streptococcus is grown in boiled milk. Thus, if we take the curve after the lag period and extrapolate it to zero time, as indicated by the dash line in Fig. 1, we find that if the surviving bacteria were grown from a single resistant organism this organism was not produced until the 5th hour.

The findings justify us in concluding that mastitis streptococci become adapted to the inhibitory agent. Further proof of this conclusion is given by Experiment 3 in which bacteria surviving the lag period are found to grow rapidly, without lag, when placed in fresh

milk. From Fig. 3 it will be seen that mastitis streptococci removed from milk cultures after various periods of incubation and placed in fresh milk behave much as if they had been left in the original culture. The $4\frac{1}{2}$ hour sample had not reached the period of rapid growth and showed a short lag in fresh milk. The streptococci removed after rapid growth showed no lag.

The results of the experiment cited raise the question as to what would occur if the bacteria were frequently placed in fresh milk during the period of incubation. This was tested in Experiment 4, the conditions of which resemble in some respects those found in the udder. In this experiment a milk culture of mastitis streptococci was diluted 1:5 with fresh (pasteurized) milk each $1\frac{1}{2}$ hours. The tabulated bacterial count (B) is corrected for the dilution (D) so that the numerical value of BD/625 represents the bacteria per cc. for all cultures, on the basis of the dilution in the last culture. Fig. 4 shows that in spite of changing the milk each $1\frac{1}{2}$ hours, the streptococcus develops a resistant type in 6 hours, whereas a lag of 4 hours occurred in the control tube the milk of which was not changed. As a result of the repeated additions of milk there was approximately a 25 per cent mortality among the streptococci. When the experiment was performed with boiled milk as a medium, rapid multiplication took place in spite of the frequent dilutions.

It has been shown[2] that scarlet fever streptococci die in milk. Their numbers gradually decline during the first 24 hours and finally the milk becomes sterile. The fact that the effect is not one of lysis is readily determined by microscopic examination, since intact streptococci may be demonstrated in the milk. It would seem that the action of milk against both the bovine and the scarlet fever streptococcus involves a factor which prevents multiplication. In the case of the mastitis streptococcus cell division is prevented for a time but a resistant type that is capable of multiplication develops. The scarlet fever streptococcus is unable to produce resistant forms and hence perishes.

Experiment 5, in which we attempted to adsorb the substance with large numbers of dead or living streptococci, indicates that

[2] Jones, F. S., *J. Exp. Med.*, 1928, **47**, 965.

there is no specific union between bacterial cell and the substance, since the inhibitory character of the milk was not diminished by such treatment. It seems probable that the substance is one preventing multiplication of bacterial cells and is not notably toxic in itself.

SUMMARY

The data here presented indicate that the inhibitory principle affecting the growth of streptococci in milk is not greatly utilized during the lag phase and that the abrupt termination of lag is not due to the utilization of the principle. They further indicate that the sudden beginning of growth cannot be ascribed to a resistant type of streptococcus present in the culture from the first, but to an adaptation occurring during the lag phase. The addition of large numbers of dead or living streptococci to milk, for limited periods, fails to diminish appreciably the inhibitory principle.

THE LESIONS IN THE SKELETAL MUSCLES IN EXPERIMENTAL SCORBUTUS

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PLATE 13

(Received for publication, June 3, 1929)

This report describes a lesion observed in the skeletal muscles of scorbutic guinea pigs and seeks to clarify its relationship to the scorbutic process as well as to demonstrate a factor modifying its distribution.

The voluminous literature bearing on scorbutus contains relatively little reference to the skeletal muscles. With two exceptions those authors who have described muscle lesions have merely mentioned that thinning and waxy degeneration of the fibers are occasionally to be found. Reports of degeneration of the muscles occur in the early studies of the disease in man by Hayem (1), Nambu and Sato (2), and recently in a study of experimental scurvy by Meyer (3).

The first report to emphasize the importance of the lesions in muscles was the monograph of Aschoff and Koch (4), containing observations of the disease in Balkan soldiers during the recent war. These authors studied the thigh muscles but were primarily interested in the hemorrhages, and chose hemorrhagic areas for study. They found changes similar to those previously reported by other workers. They believed the hemorrhages were largely responsible for the degenerative change and the thinning and atrophy the result of the cachexia in their cases.

Subsequently Höjer (5) described lesions in the muscles in experimental scorbutus which he regarded as an intrinsic part of the disease. His observations conform with our own on several points.

The material on which this report is based consists of seventy-one guinea pigs of which forty-eight were scorbutic. Many of the animals were studied in coöperation with Dr. Walter H. Eddy of Teachers College, Columbia University, who was conducting feeding experiments, and these were partially protected with canned vegetable or fruit. Most of the animals were submitted to us for diagnosis, without

record of their weight curve, behavior or diet. In some cases only selected tissues were received. The examination was chiefly of ribs and long bones, where characteristic changes occur in scurvy.

The diagnosis of scurvy is not difficult. The criteria employed were cessation of bone growth, irregularities, spreading and fragmentation of the cartilage columns and the development of the so-called "gerüstmark," a lax, delicate connective tissue which appears at the junction of bone and cartilage. Other elements in the histo-anatomical picture are hemorrhages and separation of bone and cartilage or pathological fracture of bone. The lesions are not uniformly disposed and many samples are at times necessary to estimate properly the extent of the disease or even its presence. The latter fact is especially true in animals which receive a diet only partially deficient in anti-scorbutic material.

In our routine examination of the ribs we commonly found a lesion of the intercostal muscles. Its nature varied with the characteristics of the bone lesion.

When the disease is very marked and active in the bones one finds that a variable number of muscle cells have become opaque, strongly eosinophilic and fragmented. The lines of recent fractures run directly across the fibers, paralleling the striations. Usually, however, the striations have disappeared. The segments of the muscle cells may be rectangular, square or discoid. In the latter case a series of these discoid fragments resembles blood rouleaux. Some muscle cells have been seen in which there were regular, deep clefts on only one side of the cell. The degenerate portions lose their affinity for acid dyes and appear swollen. Some show crevices and loculi within. These fibers are surrounded by large endothelial cells which engulf them and form foreign body giant cells.

Two other kinds of cells may be distinguished. Rows of spindle shaped fibroblasts with delicate fibroglia form between the muscle cells and large cells with clear acidophilic cytoplasm appear, singly and in clusters, near the point of fracture and even between the muscle fibers. These latter cells resemble young muscle cells and many are in process of mitotic division. A further study of these cells in under way to determine if possible their exact nature.

In chronic scorbutus, that is, where the lesion in the bone appears

inactive and the diseased costo-chondral junction is largely fortified by a connective tissue scar, and the "gerüstmark" has become passive, its cells no longer dividing actively, the muscles present a different picture. In such cases densely stained hyaline fragments, few in number, will be found lying in a lax, collagen-poor connective tissue. This stroma is identical with the "gerüstmark" in its cellular structure with the single exception that in the latter case dead islands of calcified cartilage or other remnants of past osteoid function are embedded within it, while in the former, fragments of muscle fibers remain.

It will be noted that in many features the lesion described resembles hyaline or Zenker's degeneration as seen in infectious and toxic diseases. Indeed the resemblance to that variety of degeneration as Forbus (6) has found it in guinea pigs is very close. Our animals were carefully searched for evidence of infectious disease but in none of those appearing in this series was there reason to suspect infection.

After some experience with this material it appeared that animals with scorbutic changes in the bones invariably presented lesions in the muscles. Indeed, in cases where the first preparations showed muscle involvement but no bone changes, further sampling regularly disclosed the presence of lesions in other bones. The intercostal muscles were invariably involved. A search for similar changes in the extensors of the thighs and forelegs and in the psoas muscle disclosed no comparable changes. Only an occasional darkly stained muscle cell was seen. It is of interest to note that in a general way within the intercostal muscles the lesions tended to favor certain locations. They were most frequent near the junction of cartilage and bone, or when present throughout the muscle they were more pronounced in these regions.

Several facts suggest that the muscle lesion is an intrinsic part of the scorbutic process. It occurs regularly in the scorbutic animals and never in the controls. It bears a definite and constant relationship to the scorbutic lesion in the bones, being active when bone changes are active, and inactive when the bone lesions are inactive. Moreover, we have not been able to find any other cause for the condition.

The selectivity of the lesion for the thoracic wall seemed to call for an explanation. It was suspected that possibly another factor other than the scurvy determined the location of the lesion and that this factor might be the activity of the thoracic muscles. The weakened

scorbutic guinea pig spares the muscles of the skeleton except those necessary to vital functions, as the respiratory muscles. If the conception just mentioned is correct the diaphragm and possibly the masseter muscles should show some degeneration as well as the intercostals. In most of our specimens these muscles had not been preserved but of eight cases in which material was available for examination the masseter muscles were found to be degenerate in three instances and the diaphragmatic muscles in five.

To further test this point of view several animals with advanced scorbutus were placed in a large barrel which could be rotated slowly with a motor so that the animals were required to exert themselves to stay on their feet. The animals were given 1 hour of this exercise every day and were then sacrificed. In each instance, florid, extensive lesions were found in the several muscles of the hind and fore legs and psoas muscles. In five non-scorbutic animals no muscle lesions were found. In all, seven scorbutic guinea pigs have been so tested. The records of three are given below.

DISCUSSION

The earliest clinical evidence of scurvy in a guinea pig is a change in attitude. At times this is represented by the elevation and sparing of one leg or by a tendency to squat quietly for long periods. Later in the disease the animals become nearly helpless and may lie on one side, apparently unable to move. In human cases an early sign of the disease (7) is loss of vigor with a tendency to tire quickly. Scorbutic infants exhibit marked tenderness in their thighs and commonly keep their extremities slightly flexed. These well known phenomena have been explained as the result of hemorrhages. The tenderness in the thighs of scorbutic infants has been said to be the result of subperiosteal hemorrhages. Observation of our guinea pigs suggests that muscle lesions may be largely responsible for both the tenderness and weakness. Moreover we have seen tenderness and the characteristic muscle degeneration develop in muscles which had been bruised.

The selective development of the muscle degeneration at points of exercise and trauma is in accord with other features of scorbutus. A causal relationship exists in the case of the hemorrhages. As was

emphasized by Aschoff and Koch, hemorrhages occur in places exposed to trauma. Bleeding about vessels and nerves was noted by these authors only when the vessel or nerve was in an exposed location, never when it was protected by bone.

A less marked relationship exists in the case of the bone lesions but here a causal connection between stress and lesion is suggested. In the thoracic wall, for example, lesions are most common in the more active and exposed ribs being relatively uncommon in the first two or three of these bones. I have noted that guinea pigs kept in separate cages, which remain quiet throughout the disease, show less marked lesions than animals which have been kept active by crowding or excitement. A special observation of the sort was made in the case of six animals sent to us while alive and which had been necessarily much shaken in transit. Some of the most striking muscle and bone lesions were present in this group.

Wolbach and Howe (8) believe that the underlying change in the bones is a deficiency in the intercellular substances. Aschoff and Koch and others have supposed that the hemorrhages were the result of altered cement substances in the vessel walls which predispose to rupture or leakage. We have noted rupture in the striated muscle. Whether a related underlying cause is responsible is not apparent from our material. We have seen cells which had ruptured but which appeared otherwise normal. If degeneration of the muscle fiber does not precede separation it follows it closely, for the usual fragmented cell is hyaline.

CONCLUSIONS

1. Muscle degeneration was constantly found in the intercostal muscles of scorbutic guinea pigs.

2. It has likewise been found in the masseter and diaphragmatic muscles.

3. Exercise will produce an identical lesion in other skeletal muscles in scorbutic animals.

4. The lesions appear to be an intrinsic part of the scorbutic process.

5. It is suggested that the tenderness over muscles in scorbutic animals and in man may be due to this myopathy.

Specimen Histories of Exercised Animals

Animal A.—Had been on basal diet plus 2 gm. canned tomato for 90 days. The weight at the beginning of the experiment was 315 gm. The weight on the 90th day was 422 gm. On the 91st and 92nd days the animal was exercised ½ hour, on the following 4 days 1 hour each day. The following day the animal was sacrificed. There was much pale fat throughout. The joints were easily dislocated, the bone crackled like egg shell, the teeth were loose. The costochondral junctions showed broad zones of "gerüstmark." A few, small, superficial hemorrhages were present in the muscles of the extremities. In the microscopic preparations all of the muscles of the extremities as well as the psoas and intercostal muscles showed extensive and severe degeneration.

Animals B and C.—Were practically identical. They were receiving 2 gm. canned peas daily and had gained weight. Both had the attitude of scorbutic animals and had joint tenderness. Each was exercised on 3 successive days for 1 hour per day. In both there was widespread muscle degeneration and abundant evidence of scorbutus in the bony system.

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EXPLANATION OF PLATE 13

Fig. 1. Degeneration in an intercostal muscle with active proliferation of young muscle cells (?). Two of these cells are in process of mitotic division. Many fibroblasts and endothelial cells are likewise present.

Fig. 2. A late stage in muscle degeneration in scorbutus. A few muscle fiber fragments lie in a collagen poor connective tissue stroma.

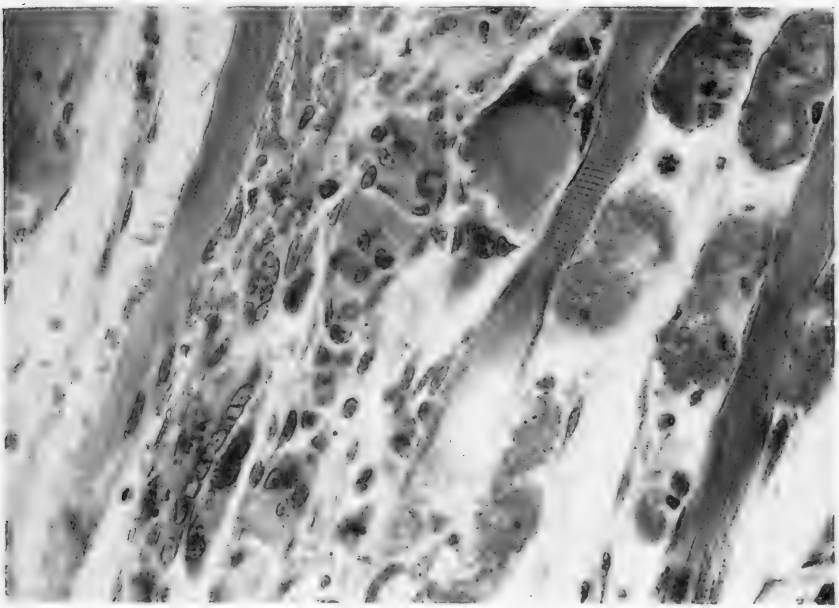

FIG. 1

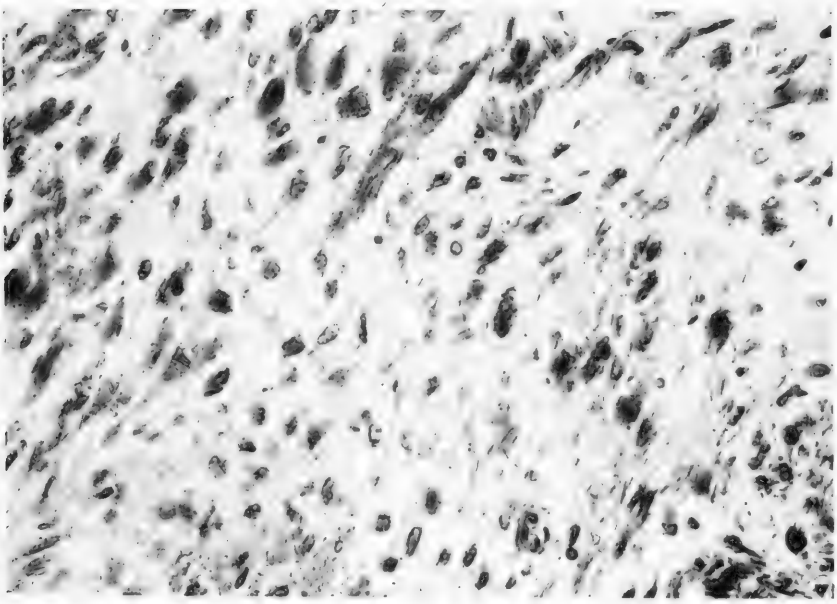

FIG. 2

(Dalldorf: Lesions in experimental scorbutus)

THE GERMICIDAL ACTION OF HYDROXY SOAPS

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This paper is the fourth of a series of papers dealing with the germicidal action of soaps and soap derivatives. In the present investigation, the α-hydroxy soaps, and the soap of ricinoleic acid, were prepared and tested.

Preparation of Hydroxy Fatty Acids

The α-hydroxy fatty acids are prepared by heating the α-brom fatty acids with an excess of dilute aqueous sodium hydroxide. This is best done by heating in the Arnold steam sterilizer for several hours. The bromine is split off by the alkali, forming sodium bromide and the hydroxy soap. A small amount of the alpha unsaturated soap is likewise formed. The hydroxy acid is liberated with dilute sulphuric acid, filtered, washed with water, and dried. One has a choice of several satisfactory methods of purification: the most convenient is to dissolve the product in a small quantity of ether and then add a large excess of petroleum ether, which precipitates the hydroxy acids. This process is repeated until a constant melting point is obtained. Further details for the preparation and purification of the various hydroxy acids are given by the following authors: α-hydroxybehenic acid, by Fileti (1907); α-hydroxystearic, -palmitic, and -myristic acids, by Le Sueur (1905); α-hydroxylauric acid, by Guerin (1903); α-hydroxycapric acid, by Bagard (1907); and α-hydroxycaprilic acid, by Ley (1877). The hydroxy acid with 20 carbon atoms was not prepared. (In the preceding investigation (Eggerth, 1929), the α-bromoarachidic acid was prepared and tested, on the assumption that it was a normal acid having 20 carbon atoms. The writer at that time was not acquainted with the work of Ehrenstein and Stuewer (1922) who have shown that arachidic acid is actually iso-behenic, with 22 carbon atoms. It is interesting to note that the α-bromoarachidic soap actually gave germicidal titers that were in every case practically the same as those of the α-bromobehenate.)

The α-hydroxy fatty acids have higher melting points and are stronger acids than the parent unsubstituted acids. Their soaps are somewhat less hydrolysed by water. Nevertheless, those soaps having 14 or more carbon atoms are strongly alkaline to phenolphthalein. The sodium soaps are very little soluble in water; the potassium soaps are much more so.

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Ricinoleic acid is a normal fatty acid having 18 carbon atoms, with a double bond between the 9th and 10th carbon atoms, and a hydroxyl group attached to the 12th carbon atom. It is a hydroxylated oleic acid. It seemed desirable to study it here, inasmuch as it is the only member of its series that is known and available. It was prepared from castor oil by saponification; the liberated crude acid was thoroughly extracted with petroleum ether, converted to the barium salt, recrystallized several times from alcohol, and liberated with sulphuric acid.

Parallel tests were made with soaps of saturated unsubstituted fatty acids and oleic acid. The former were purchased from the Eastman Kodak Co.; the latter was Kahlbaum's best grade of oleic acid.

Technic of Germicidal Tests

The potassium soaps were made by adding the theoretical amount of KOH solution to weighed quantities of the fatty acids. Serial dilutions were made in sterile distilled water just prior to making the germicidal tests.

Buffer solutions set at pH 6.5, 7.5, and 8.5 were prepared. These contained N/10 potassium phosphate and N/20 glycine; details of their composition are given in a previous paper (Eggerth, 1926). When the test organism was *Diplococcus pneumoniæ*, the buffer solution was made to contain N/20 phosphate. The sterilized buffer fluids were inoculated with the test organisms, and 0.5 cc. quantities were pipetted into series of small test tubes; to these, 0.5 cc. quantities of the soap dilutions were added and the contents of the tubes well mixed. Thus the final concentration of potassium phosphate was N/20, except in the case of *Diplococcus pneumoniæ*, where the final concentration was N/40. (*Diplococcus pneumoniæ* frequently failed to survive for 18 hours in the N/20 potassium phosphate controls, whereas it always survived in the N/40 phosphate.) The tests were incubated at 37°C. and one loopful subcultured on glucose blood agar plates at the end of 30 minutes, 2 hours, and 18 hours.

The following test organisms were used: *Diplococcus pneumoniæ; Streptococcus hæmolyticus; B. diphtheriæ; Staphylococcus aureus; Micrococcus ovalis; B. typhosus; Vibrio choleræ; B. lepisepticus; B. melitensis; B. pyocyaneus*. With the exception of the last, these organisms are the same strains as those used in previous investigations with soaps (Eggerth, 1926, 1927, 1929).

The inoculations in the germicidal tests were such that each cubic centimeter of final test fluid contained 0.02 cc. of a 24 hour broth culture, with the exception of *Staphylococcus aureus*, *B. typhosus*, and *B. pyocyaneus*, where the inoculum was 0.01 cc.

DISCUSSION

As with other soaps previously reported, the germicidal titers of the α-hydroxy soaps increase with increasing molecular weight to a

TABLE I

The Germicidal Titers of α- Hydroxy-Soaps

	α-hydroxy-caprylate	α-hydroxy-caprate	α-hydroxy-laurate	α-hydroxy-myristate	α-hydroxy-palmitate	α-hydroxy-stearate	α-hydroxy-behenate
No. of carbon atoms	8	10	12	14	16	18	22
Diplococcus pneumoniæ							
Time / pH							
2 hrs. 6.5	0	N/160	N/2560	N/20,480	N/163,840	N/163,840	
7.5	0	N/40	N/320	N/2560	N/10,240	N/5120	0
8.5	0	N/40	N/320	N/2560	N/10,240	N/2560	
18 hrs. 6.5	N/20	N/640	N/5120	N/81,920	N/327,680	N/327,680	
7.5	N/20	N/80	N/1280	N/5120	N/40,960	N/40,960	N/20,480
8.5	N/20	N/80	N/1280	N/2560	N/40,960	N/20,480	
Streptococcus hæmolyticus							
2 hrs. 6.5	0	N/20	N/320	0	0	0	
7.5	0	N/20	N/160	N/320	N/160	N/80	0
8.5	0	0	N/160	N/640	N/160	N/160	
18 hrs. 6.5	0	N/320	N/640	N/2560	N/5120	N/640	
7.5	0	N/40	N/640	N/1280	N/1280	N/640	N/640
8.5	0	N/40	N/640	N/1280	N/1280	N/640	
Staphylococcus aureus							
2 hrs. 6.5	0	0	N/2560	N/10,240	N520	N/640	
7.5	0	N/10	N/160	N/80 N/20,480	N/80	N/80	0
8.5	0	0	N/40	N/320	N/160	N/80	
18 hrs. 6.5	0	N/40	N/5120	N/40,960	N/20,480	N/1280	
7.5	0	N/20	N/640	N/160 N/20,480	N/160 N/10,240	N/1280	0
8.5	0	0	N/160	N/640	N/1280	N/640	
B. diphtheriæ							
2 hrs. 6.5	0	N/160	N/2560	N/20,480	N/20,480	N/320	
7.5	0	N/20	N/160	N/80	N/80	N/80	0
8.5	0	N/20	N/160	N/320	N/640	N/320	
18 hrs. 6.5	0	N/640	N/10,240	N/81,920	N/81,920	N/40,960	
7.5	0	N/80	N/1280	N/20,480	N/40,960	N/20,480	0
8.5	0	N/20	N/320	N/2560	N/5120	N/1280	

TABLE I—*Continued*

		α-hydroxy-capry-late	α-hydroxy-caprate	α-hydroxy-laurate	α-hydroxy-myristate	α-hydroxy-palmitate	α-hydroxy-stearate	α-hydroxy-behenate
No. of carbon atoms		8	10	12	14	16	18	22
				Micrococcus ovalis				
Time	pH							
2 hrs.	6.5	0	0	N/160	0	0	0	
	7.5	0	0	N/80	N/80	0	0	0
	8.5	0	0	N/80	N/160	N/320	0	
18 hrs.	6.5	0	N/40	N/320	N/1280	N/1280	0	
	7.5	0	N/20	N/160	N/160	N/320	0	0
	8.5	0	0	N/160	N/640	N/1280	0	
				Vibro choleræ				
2 hrs.	6.5	0	N/160	N/2560	N/5120	N/320	0	
	7.5	0	N/40	N/320	N/1280	N/640	0	0
	8.5	0	N/40	N/160	N/1280	N/640	N/160	
18 hrs.	6.5	N/20	N/640	N/5120	N/5120	N/640	0	0
	7.5	0	N/40	N/640	N/2560	N/640	N/160	0
	8.5	0	N/40	N/640	N/2560	N/640	N/160	
				B. lepisepticus				
2 hrs.	6.5	0	N/320	N/5120	N/20,480	N/5120	N/320	
	7.5	0	N/80	N/320	N/1280	N/2560	N/640	N/160
	8.5	0	N/80	N/160	N/1280	N/5120	N/640	
18 hrs.	6.5	N/40	N/640	N/10,240	N/81,920	N/10,240	N/2560	
	7.5	N/20	N/320	N/1280	N/2560	N/2560	N/1280	N/640
	8.5	N/10	N/160	N/640	N/2560	N/5120	N/2560	
				B. melitensis				
2 hrs.	6.5	0	N/80	N/320	N/2560	0	0	
	7.5	0	N/40	N/160	N/1280	N/80	0	0
	8.5	0	N/40	N/160	N/1280	N/320	0	
18 hrs.	6.5	N/20	N/160	N/2560	N/5120	N/5120	0	
	7.5	N/10	N/160	N/640	N/1280	N/160	0	0
	8.5	N/10	N/80	N/320	N/1280	N/1280	0	

TABLE I—*Concluded*

		α-hydroxy-caprylate	α-hydroxy-caprate	α-hydroxy-laurate	α-hydroxy-myristate	α-hydroxy-palmitate	α-hydroxy-stearate	α-hydroxy-behenate
No. of carbon atoms		8	10	12	14	16	18	22
B. pyocyaneus								
Time	pH							
2 hrs.	6.5	0	0	0	0	0	0	
	7.5	0	0	N/80	N/160	N/80	0	0
	8.5	0	0	N/80	N/640	N/1280	0	
18 hrs.	6.5	0	0	0	0	0	0	
	7.5	0	0	N/80	N/160	N/80	0	0
	8.5	0	0	N/80	N/2560	N/1280	0	
B. typhosus								
2 hrs.	6.5	0	N/40	N/160	0	0	0	
	7.5	0	N/10	N/160	N/640	N/80	0	0
	8.5	0	N/40	N/160	N/1280	N/320	0	
18 hrs.	6.5	0	N/40	N/320	0	0	0	
	7.5	0	N/40	N/160	N/640	N/160	N/80	0
	8.5	0	N/40	N/320	N/2560	N/1280	N/160	

The lowest dilutions tested were N/10 for the hydroxycaprylate and hydroxycaprate, N/40 for the hydroxylaurate, and N/80 for the others. Temperature, 37°C.

given maximum, and then diminish. (Table I and Figs. 1 and 2.) With all organisms, this maximum occurs either with the a-hydroxymyristate or the -palmitate; there is no sharp differentiation between Gram positive and Gram negative bacteria as was the case with the α-brom soaps (Eggerth, 1929).

The germicidal action of the α-hydroxy soaps varies a great deal with the different test organisms (see Table I). The α-hydroxycaprylate does not kill any organism in a concentration of N/10 in 2 hours; in 18 hours, slight germicidal action is manifested towards *Diplococcus pneumoniæ*, *B. melitensis*, and *B. lepisepticus*. The α-hydroxycaprate is more active, especially toward *Diplococcus pneumoniæ*, *B. melitensis*, *B. lepisepticus*, *Vibrio choleræ*, and *B. typhosus;* there is no action upon *B. pyocyaneus*, and very little upon *Staphyloccus aureus* and *Micrococcus ovalis*. The α-hydroxylaurate acts more

uniformly toward the ten test organisms than the other soaps of this series; the highest titers at pH 7.5 for 2 hours being N/320 and the lowest, N/80. Germicidal titers increase with the α-hydroxy-myristate, though very unequally. As we pass to the α-hydroxypalmitate, we find that the titers are increased with *Diplococcus pneumoniæ* and *B. lepisepticus*, but diminished with *Streptococcus hæmolytics*, *Vibrio choleræ*, *B. melitensis; B. pyocyaneus*, and *B. typhosus*. The α-hydroxystearate is still highly germicidal for *Diplococcus pneumoniæ*, *B. diphtheriæ*, and *B. lepisepticus;* its titer for the other organisms is

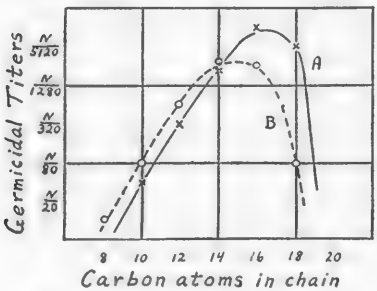

FIG. 1. The germicidal titers of α-hydroxy soaps (*A*) and of unsubstituted saturated soaps (*B*) for *Diplococcus pneumoniæ*, at pH 7.5. The soaps are designated by the number of carbon atoms in their molecule. Time of test, 2 hours; temperature, 37°C.

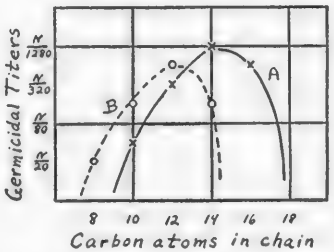

FIG. 2. The germicidal titers of α-hydroxy soaps (*A*) and of unsubstituted saturated soaps (*B*) for *Vibrio choleræ*, at pH 7.5. The soaps are designated by the number of carbon atoms in their molecule. Time of test, 2 hours; temperature, 37°C.

very much diminished. Curiously enough, the only germ to be killed by the α-hydroxybehenate in 2 hours was *B. lepisepticus;* in 18 hours, this soap was toxic for three species: *Diplococcus pneumoniæ*, *Streptococcus hæmolyticus*, and *B. lepisepticus*.

The specific effect of the hydroxyl group upon the germicidal titer may be seen on comparing Table I with Table II, and in Figs. 1 and 2. In many cases, though by no means all, the germicidal titers of an α-hydroxy soap are very nearly the same as that of an unsubstituted soap having two less carbon atoms. This is particularly true of the titers for *Vibrio choleræ*, *B. melitensis*, *B. lepisepticus*, and *Strepto-*

TABLE II

The Germicidal Titers of Unsubstituted Soaps, at pH 7.5

	Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Stearate
No. of carbon atoms	6	8	10	12	14	16	18
				Diplococcus pneumoniæ			
2 hrs.	0	N/10	N/80	N/640	N/2560	N/2560	N/80
18 "	N/10	N/20	N/160	N/1280	N/10,240	N/10,240	N/160
				Streptococcus hæmolyticus			
2 hrs.	0	N/10	N/40	N/320	N/640	N/160	0
18 "	0	N/20	N/80	N/640	N/1280	N/640	0
				Staphylococcus aureus			
2 hrs.	0	0	N/40	N/320	N/160	0	0
18 "	0	0	N/40	N/320	N/640	0	0
				B. diphtheriæ			
2 hrs.	0	0	N/40	N/640	N/320	N/80	0
18 "	0	N/10	N/160	N/1280	N/5120	N/80	N/80
				Micrococcus ovalis			
2 hrs.	0	0	N/20	N/80	N/40	0	0
18 "	0	0	N/80	N/320	N/40	0	0
				Vibrio choleræ			
2 hrs.	0	N/20	N/160	N/640	N/160	0	0
18 "	N/20	N/20	N/320	N/640	N/320	0	0
				B. lepisepticus			
2 hrs.	0	N/10	N/160	N/1280	N/2560	N/160	0
18 "	0	N/40	N/640	N/10,240	N/10,240	N/320	0
				B. melitensis			
2 hrs.	0	N/10	N/160	N/1280	N/320	0	0
18 "	0	N/20	N/160	N/2560	N/1280	0	0

TABLE II—*Concluded*

	Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Stearate
No. of carbon atoms	6	8	10	12	14	16	18
			B. pyocyaneus				
2 hrs.	0	0	0	N/20	N/40	0	0
18 "	0	0	0	N/20	N/40	0	0
			B. typhosus				
2 hrs.	0	N/10	N/40	N/160	0	0	0
18 "	0	N/10	N/80	N/160	0	0	0

The lowest dilutions tested were N/10 for the caproate, caprylate, and caprate, N/20 for the laurate, N/40 for the myristate and N/80 for the palmitate and stearate. Temperature, 37°C.

coccus hæmolyticus. If the germicidal titers are plotted against the number of carbon atoms in the soap molecule, as in Figs. 1 and 2, it will be seen that the effect of hydroxylation is to shift the curve to the right.

In addition to this general effect, specific effects varying a great deal with the test organisms may be observed. This is best brought out by computing the ratios between the titers of an unsubstituted soap, such as potassium myristate, and the corresponding α-hydroxymyristate. This is done in Table III, which is condensed from Tables I and II. For comparison, the titers for potassium α-bromomyristate from a previous paper (Eggerth, 1929) are included. The last column in Table III shows that the effect of introducing a hydroxyl group (or bromine atom) is not uniform, but varies widely with the species of organism. Table III shows that the hydroxylation of myristic acid greatly increases the germicidal action for *B. typhosus*, *B. pyocyaneus*, *B. melitensis*, *Vibrio choleræ*, and *Staphylococcus aureus* (second value); it diminishes the 2 hour titer for *B. diphtheriæ* (but, as shown in Table I, it greatly increases the 18 hour titer); the titers for the other organisms are practically unchanged. It is to be noticed that the effects of hydroxylation are often quite different from those of bromination (Table III).

With the unsubstituted soaps, the 18 hour titer is in most cases double that of the 2 hour titer (Table II); with the hydroxy soaps, the 18 hour titer is often much greater than that for 2 hours. The most striking example of this is *B. diphtheriæ* with the α-hydroxymyristate, -palmitate, and -stearate, where, at pH 7.5 the 18 hour titer is 256 times as great as the 2 hour titer (Table I).

With one organism, *Staphylococcus aureus*, a very peculiar result was quite consistently obtained. With the α-hydroxymyristate and -palmitate, this germ gave two distinct zones of germicidal action at

TABLE III

The Germicidal Titers of Potassium Myristate, Potassium α-Hydroxymyristate, and Potassium α-Bromomyristate

Test organism	Myristate	α-hydroxy-myristate	α-bromo-myristate	Ratio
Diplococcus pneumoniæ	N/2560	N/2560	N/40,960	1:1:16
Streptococcus hæmolyticus	N/640	N/320	N/10,240	2:1:32
Staphylococcus aureus	N/160	N/80 N/20,480	N/5120	2:1:64 1:128:32
B. diphtheriæ	N/320	N/80	N/5120	4:1:64
Micrococcus ovalis	N/40	N/80	N/2560	1:2:64
Vibrio choleræ	N/160	N/1280	N/1280	1:8:8
B. melitensis	N/320	N/1280	N/2560	1:4:8
B. lepisepticus	N/2560	N/1280	N/5120	2:1:4
B. pyocyaneus	N/40	N/160	N/20	2:8:1
B. typhosus	0	N/640	N/20	0:16:1

All tests were made at pH 7.5; temperature, 37°C.

pH 7.5 (Table I). Thus, with the hydroxymyristate, concentrations of N/40 and N/80 were germicidal in 2 hours; concentrations of N/160 to N/2560 gave growth on subculture (though often with a much diminished number of colonies); concentrations of N/5120, N/10,240, and N/20,480 were again usually germicidal, though occasionally one to five colonies appeared on the subculture. (A loopful of the control, when diluted and plated out, was shown to give at least 15,000 colonies). Higher dilutions showed no germicidal action. When the time of test was increased to 18 hours, the results were usually still the same, though occasionally all concentrations up to N/20,480 were found to be germicidal. These two zones appeared likewise with the α-

hydroxypalmitate, but not with any other soap. They appeared only at pH 7.5. With *B. diphtheriæ*, a similar phenomenon was occasionally observed with the same soaps, but it appeared so irregularly that it was not indicated in Table I.

The above phenomenon appeared so extraordinary that a number of experiments were undertaken to verify and explain it. These may be summarized as follows:

1. Two other strains of *Staphylococcus aureus*, recently isolated from pus, showed the same phenomenon.

2. Colonies from *Staphylococci* that survived an α-hydroxymyristate concentration of N/320 for 18 hours were fished and tested. They gave practically the same titers as the original stock culture. The resisting forms are, therefore, not mutants.

3. Mixed inoculations of *Staphylococcus aureus* and *Streptococcus hæmolyticus* were tested with α-hydroxymyristate at pH 7.5. Concentrations of N/160 and N/320 gave, on subculture, pure cultures of *Staphylococcus aureus;* concentrations of N/5120, N/10,240, and N/20,480 gave pure cultures of *Streptococcus hæmolyticus*. Mixed inoculations of *Staphylococcus aureus* with *Diplococcus pneumoniæ*, *Vibrio choleræ*, *B. lepisepticus*, *B. melitensis*, and *B. typhosus* gave similar results.

4. *Staphylococci* were grown on agar and emulsified in sterile buffer in such quantity that the fluid was faintly turbid. Dilutions of α-hydroxymyristate were added as usual. With this large inoculum, organisms could be found with ease in stained films. Subcultures of all dilutions were still positive after 24 hours. After 48 hours, subcultures from soap dilutions of N/160 to N/5120 were negative; films made at the same time showed large numbers of well staining, unagglutinated *Staphylococci*. This experiment rules out agglutination of the organisms by higher dilutions of the soap as a possible cause of the phenomenon.

5. Bacteriostatic tests were made in fluid media. These tests were complicated by the fact that both peptone and meat infusion markedly inhibit the germicidal and growth inhibiting action of the α-hydroxy soaps. In this respect, these soaps differ decidedly from the unsubstituted soaps and sodium oleate (Eggerth, 1927), upon which peptone has no inhibiting action. However, it was found that *Staphylococcus aureus* grows well in a medium having the following composition: N/40 potassium phosphate at pH 7.5; 0.03% Parke Davis peptone, and 0.3% glucose. This small amount of peptone is only slightly inhibitory to these soaps. After 3 days at 37°C. stained films were made from the sediment of each tube, and phenol red was added to determine acid production; these two tests for growth always confirmed one another. The bacteriostatic concentrations of these soaps for *Staphylococcus aureus* were found to be: N/640 for α-hydroxylaurate; N/10,240 for α-hydroxymyristate and -palmitate; N/2560 for α-hydroxystearate. Two other recently isolated *Staphylococci* gave the same results.

6. Instead of subculturing one loopful in the usual way, quantities of 0.1, 0.01, and 0.001 cc. of test fluids were plated out and colony counts made. The results are given in Table IV. This experiment shows that concentrations of the α-hydroxymyristate of N/20,480 or more, rapidly kill off the great majority of the *Staphylococci* present. There are, however, a few survivors in concentrations less than N/80, and the survivors are more numerous in the zone from N/160 to N/1280 than in zone from N/2560 to N/20,480.

It is remarkable that some organisms in a culture can tolerate a concentration of germicide that is 128 times as great as that which

TABLE IV

Colony Counts of Staphylococcus aureus Treated with Potassium α-Hydroxymyristate

Concentration of soap	Number of colonies per cubic centimeter	
	After 2 hrs.	After 18 hrs.
N/80	0	0
N/160	5000	0
N/320	12,000	650
N/640	30,000	3500
N/1280	80,000	5000
N/2560	50	10
N/5120	80	0
N/10,240	30	0
N/20,480	200	250
N/40,960	Innumerable	Innumerable
N/81,920	"	"
Control	"	"

This test was made at pH 7.5; temperature, 37°C.

is lethal to the great bulk of the bacteria in the culture. When the number of survivors is reduced to 100 or less per cubic centimeter a loopful, when cultured, is likely not to give any growth at all, thus giving the second germicidal zone.

The experiment shown in Table IV was repeated several times; the actual colony counts varied a great deal in different experiments, showing that the number of soap resistant organisms in a culture varied greatly. The comparative results, however, remained essentially the same. Attempts were made to determine what factors influenced the proportion of resistant organisms in a culture, but without suc-

TABLE V

The Germicidal Titers of Oleates and Ricinoleates

pH	Oleate		Ricinoleate	
	2 hrs.	18 hrs.	2 hrs.	18 hrs.
		Diplococcus pneumoniæ		
6.5	N/327,680	N/327,680	N/20,480	N/81,920
7.5	N/40,960	N/327,680	N/5120	N/20,480
8.5	N/20,480	N/163,840	N/2560	N/20,480
		Streptococcus hæmolyticus		
6.5	N/163,840	N/327,680	N/10,240	N/20,480
7.5	N/20,480	N/40,960	N/2560	N/10,240
8.5	N/10,240	N/40,960	N/1280	N/5120
		B. diphtheriæ		
6.5	N/81,920	N/163,840	N/10,240	N/20,480
7.5	N/20,480	N/40,960	N/1280	N/2560
8.5	N/10,240	N/20,480	N/320	N/1280
		Staphylococcus aureus		
6.5	0	0	N/2560	N/5120
7.5	0	0	N/640	N/1280
8.5	N/40	N/80	N/640	N/1280
		Micrococcus ovalis		
6.5	0	0	0	0
7.5	0	N/40	N/160	N/320
8.5	N/40	N/160	N/80	N/80
		Vibrio choleræ		
6.5	0	0	N/80	N/320
7.5	0	N/80	N/320	N/320
8.5	0	N/640	N/160	N/320
		B. lepisepticus		
6.5	0	0	N/5120	N/20,480
7.5	N/40	N/80	N/2560	N/10,240
8.5	N/640	N/2560	N/1280	N/5120

TABLE V—*Concluded*

pH	Oleate		Ricinoleate	
	2 hrs.	18 hrs.	2 hrs.	18 hrs.
		B. melitensis		
6.5	0	0	N/2560	N/5120
7.5	0	0	N/1280	N/1280
8.5	N/80	N/160	N/640	N/640
		B. pyocyaneus		
6.5	0	0	0	0
7.5	0	0	0	0
8.5	0	0	0	0
		B. typhosus		
6.5	0	0	0	0
7.5	0	0	0	N/40
8.5	0	0	0	N/80

The lowest concentrations tested were N/40. Temperature, 37°C.

cess; such factors as the age or previous history of the culture, or even the composition of the culture medium, seemed to be without influence.

The effect of the pH upon the germicidal action of the α-hydroxy soaps is, in general, the same as upon other soaps (Eggerth, 1926, 1929). Two main types of pH effect may be observed. First, what might be called the "normal pH effect" is illustrated by all of these soaps with *Diplococcus pneumoniæ;* the titer is highest in acid reactions, and diminishes with increasing alkalinity. Second, with might be called the "reversed pH effect," as in the case of *B. typhosus* with the α-hydroxymyristate; germicidal action is lacking at pH 6.5, but appears and increases with increasing alkalinity. In some cases a combination of the two effects is evident. The "reversed pH effect" is to be ascribed to the fact that the soaps are too insoluble at the acid pH to get a germicidal concentration into solution. In certain cases, the pH effect is of the "reversed" type for the 2 hour period, and of the "normal" type for the 18 hours period, as for *Streptococcus hæmolyticus* with α-hydroxymyristate and -palmitate (Table I). In the

latter cases it is obvious that enough soap went into solution at pH 6.5 to kill in 18 hours, but not enough to kill in 2 hours.

The effect of introducing a hydroxyl radical into an unsaturated soap is shown in Table V. One is struck by the fact that where the oleate titers are high, those for the ricinoleate are much lower; where the oleate titers are low, the corresponding ones for the ricinoleate are higher (except for *B. pyocyaneus*). The hydroxyl group in this unsaturated soap, therefore diminishes *selective* germicidal action; whereas the hydroxyl group in saturated soaps rather increases selective germicidal action.

It will be noticed that the titers of the oleate for *Streptococcus hæmolyticus* and *B. diphtheriæ* are somewhat higher than those reported for the same two organisms several years ago (Eggerth, 1926 and 1927). This seems to be due to an actual increased susceptibility of these two cultures, as several samples of Kahlbaum's oleic acid and sodium oleate were tried, and all gave the same high titers.

The Selective Germicidal Action of Soaps

It has been known for some time that the unsaturated soaps, such as the oleates, will destroy certain organisms such as *Diplococcus pneumoniæ* and *Streptococcus* in high dilutions, and yet have very little toxicity for other species, as *Staphylococcus* (Avery, 1918). Walker (1924) has more recently shown that the saturated soaps, especially the laurate, likewise show considerable selective germicidal action. The writer has tested ten organisms against three complete series of soaps, and has been repeatedly impressed by the fact that all of these soaps show selective germicidal action, though no two of them in exactly the same way. That being the case, it should be possible, by selecting the right soap, to kill at will any one of a mixture of organisms. This was actually done in a number of instances. Thus, in a mixture of *Streptococcus hæmolyticus*, *Staphylococcus aureus*, *B. diphtheriæ*, and *B. typhosus*, the soap potassium α-bromostearate in concentrations of N/40,960 and N/81,920 killed only the *Streptococcus* in 2 hours at pH 7.5; the α-hydroxymyristate in concentrations of N/5120, N/10,240, and N/20,480 killed only the *Staphylococcus* at pH 7.5; the α-hydroxystearate in concentrations of N/2560 to N/40,960 killed only *B. diphtheriæ* in 18 hours at pH 7.5; while the α-hydroxy-

myristate at pH 8.5 killed only *B. typhosus* in a concentration of N/1280. Other combinations of four organisms could not be arranged so successfully, but numerous two-organism combinations were tested with the desired results. Thus N/5120 and N/10,240 potassium laurate kills *B. lepisepticus* at pH 7.5 in 18 hours, but not *Diplococcus pneumoniæ;* whereas potassium oleate kills the latter in high dilutions, but not the former. In a mixture of *Streptococcus* and *Vibrio choleræ*, oleate or α-bromostearate destroys the *Streptococcus;* α-hydroxymyristate, the *Vibrio*. In a mixture of *Micrococcus ovalis* and *B. pyocyaneus*, α-bromostearate kills the former; α-hydroxymyristate at pH 8.5, the latter. Other similar combinations are possible.

As soon as data on other soaps and other organisms is obtained, it is likely that even more striking examples of selective germicidal action will be encountered. The possibilities are interesting.

SUMMARY AND CONCLUSIONS

1. The α-hydroxy soaps exhibit a high germicidal action toward certain organisms. As with other soaps, the germicidal action increases with molecular weight to a maximum, then diminishes. The pH effects the germicidal action as it does other soaps.

2. Certain α-hydroxy soaps give two distinct germicidal zones with *Staphylococcus aureus*.

3. The effect of the hydroxyl group in saturated soaps is to increase selective germicidal action; the effect of the hydroxyl group in an unsaturated soap is to diminish it.

4. The soaps offer a means of separating mixtures of organisms by selective germicidal action.

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PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

I. The Specific Fixation by Tissues of Susceptible Animals*

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The discovery that a group of malignant tumors of the fowl could be transmitted by a cell-free extract or a desiccate of the tumor has lead to a great diversity of opinion, not only as to the classification of the tumor group, but also as to the nature of the agents possessing the tumor producing property. By many these neoplasms are classed among the virus diseases. However there are certain biological properties of these tumors and some experimental data which are difficult to reconcile with the theory that the agents are similar to those causing the virus type of infectious disease process.

Various attempts have been made to devise means of distinguishing between the filter-passing virus group, presumably living organisms, and the active substances, products of living cells, the enzyme-like group of agents. But as far as the chicken tumors are concerned, it cannot be considered that any of the earlier studies offered very definite information as to the character of the causative agents.

Certain biological agents such as bacteriophage, enzymes, toxins and antibodies, all more or less selective in the cells or substances acted upon, are first adsorbed or fixed, and sometimes apparently inactivated by the specific substratum, while non-specific cells or substances are without effect on the agents. For example, tetanus toxin, which has a selective action on the nervous system, is neutralized *in vitro* by nervous tissue from susceptible animals, while kidney, spleen and other organs from the same animals have little or no effect (1). Furthermore, the brain tissue of animals non-susceptible to tetanus toxin has practically no neutralizing effect *in vitro* on the poison (2) (3).

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There are some indications that the chicken tumor agent may be bound by tissues under certain conditions. Pentimalli (4) observed that the activity of a chicken tumor filtrate was reduced by contact with chick embryo pulp, and also by the repair tissue from a healing wound in chickens, but in a lesser degree. Leucocytes had no activity in this respect. Deelman reports a similar observation (5).

The present work has to do with a systematic study of the action of tissues of susceptible fowls on the chicken tumor agent as compared with tissues of non-susceptible animals. A preliminary note on the subject has already been published (6).

Experimenal Method

The tumor filtrates were prepared by grinding about 5 gms. of tumor tissue with sand and then adding 100 cc. of Ringer's solution. After a thorough shaking the suspension was centrifuged to remove the sand and larger particles, and the supernatant fluid passed through a Berkefeld V filter. The tissues to be tested were ground to a fine pulp in a mortar with the addition of Ringer's solution in the ratio of about 2 gms. of tissue to 1 cc. of fluid. A measured amount of the resulting pulp was placed in a centrifuge tube and a measured amount of the Berkefeld filtrate of the tumor was added and mixed thoroughly. After a period of contact, either at room temperature or in the incubator, the mixture was centrifuged and the activity of the supernatant fluid was tested by intradermal injections in normal chickens. The activity of each filtrate was tested by the injection of an untreated sample in a similar manner.

Preliminary Experiment:—Five preliminary experiments were undertaken on a small number of animals in order to gain some idea as to the quantitative relationships and the degree of specificity of the reaction. As the technique was almost identical in the several experiments, the results are presented in tabulated form in Table I.

From these preliminary tests it would seem that the muscle of susceptible chickens definitely reduces the amount of activity of the tumor agent in the filtrate. On the other hand, the brain and liver from the same chickens, and muscle, brain and liver from rabbits have no detectable effect on the potency of the tumor agent.

Fixation and Inactivation Experiments with Muscle from Chicken, Rabbit and Pigeon:—The next step was to duplicate the above experiments on an animal more closely related to the chicken, namely the pigeon, using larger amounts of tumor filtrate.

Eight cc. of the muscle pulps were mixed with 4 cc. of fresh

TABLE I

	Mixtures	Time of contact	Resultant tumor from supernat. fluid
Exp. 1.	9 cc. chicken muscle pulp + 1 cc. tumor filtrate	3 hrs. at 31°	+
	9 cc. rabbit " " + 1 cc. " "		++++
Exp. 2.	8 cc. chicken " " + 3 cc. " "	1 hr. at 37°	++
	8 cc. rabbit " " + 3 cc. " "		++++
	3 cc. chicken brain " + 1 cc. " "		++++
	3 cc. rabbit " " + 1 cc. " "		++++
Exp. 3	8 cc. chicken muscle " + 2 cc. " "	3 hrs. at 37°	−
	8 cc. rabbit " " + 2 cc. " "	—	++++
	8 cc. chicken liver " + 2 cc. " "	1 hr. at room temp.	++++
	8 cc. rabbit " " + 2 cc. " "		++++
	3 cc. chicken brain " + 0.8 cc. " "		++++
	3 cc. rabbit " " + 0.8 cc. " "		++++
Exp. 4	8 cc. chicken muscle " + 2 cc. " "	3 hrs. at 37°	−
	8 cc. rabbit " " + 2 cc. " "	1 hr. at room temp.	++
Exp. 5	8 cc. chicken " " + 2 cc. " "	5 hours	−
	8 cc. rabbit " " + 2 cc. " "		++++

tumor filtrate and allowed to remain in contact at room temperature for 3 hours for Experiment 12 and 14, and 4 to 6 hours for all the others. One cc. of the supernatant fluids was injected intracutaneously, and a similar amount of the tissue pulp in the breast muscle of normal chickens. The results are given in Table II.

TABLE II

Exp. no.	Tumors from supernatant fluids 1 cc.		Tumors from pulps 1 cc.		Tumors from filtrate alone	
	Chicken muscle	Rabbit muscle	Chicken muscle	Rabbit muscle		
6	− −	++++ ++++	−	++++	0.5 cc. ++++	1 cc. ++++
7	++ +++ ++	++ ++++ +++	+++ +++ +	+ +++ ++	0.5 cc. ++ 5 cc. ++++	1 cc. +++ 10 cc. ++++
		Pigeon muscle		Pigeon muscle		
8	−	−	−	+++	1 cc. +++	2 cc. ++++
9	− + −	− +++ −	− −	− −	0.5 cc. −	1 cc. −
10	++ +	+++ ++++	+++ +++	+++ ++++	0.5 cc. +++ 2 cc. +++	1 cc. +++ 4 cc. ++++
11	− +	++++ ++++	− +++	− +++	1 cc. +++ 5 cc. ++++	2 cc. ++++ 5 cc. ++++
12	++ −	+++ −	++++ −	+++ −	1 cc. ++++ 2 cc. −	0.5 cc. +++ 4 cc. −
13	+++ ++++ ++++	+++ +++ ++++	++ ++++	+++ ++	0.5 cc. ++++	1 cc. ++++
14	++ ++++ ++++	++ +++ ++++	++++ ++	++++ ++	1 cc. ++	5 cc. +++

It will be noted that more tumor filtrate was used with the tissue pulp in this group of experiments than in the first group. Out of the nine experiments five gave evidence of reduction in activity of the filtrate after contact with chicken muscle pulp, while one was negative, and three showed no evidence of reduction in activity of the filtrate by chicken muscle contact. With the exception of No. 6, and probably No. 8, the injection of the pulps, both from the chicken muscle and the controls, showed them to be of about equal potency in the production of tumors.

The fact that a proportion of these experiments failed to show any marked reduction in the activity of the filtrate in contact with muscle pulp from susceptible animals suggested the possibility that too active a filtrate had been used. To test this several dilutions of the filtrate were utilized in the next group of experiments. For the results see Table III.

TABLE III

Exp. no.	Tumors from supernatant fluids 1 cc.			Tumors from pulps 1 cc.	
	Chicken muscle	Rabbit muscle		Chicken muscle	Rabbit muscle
15	++ ++	+++ +++	Pure Filtrate	++++ ++	++++ +++
	+ +	+++ ++	1:1 Filtrate	++ +++	++++ ++++
	− ?	++ ?	1:2 Filtrate	+++ ++	++++ ++++
	Chicken muscle	Pigeon muscle		Chicken muscle	Pigeon muscle
16	− −	++ ++++	Pure Filtrate	?	?
	+ −	++ +++	1:1 Filtrate	++++	++++
	+ −	+++ +++	1:2 Filtrate	++	+++
	+ +	+++ ++	1:3 Filtrate	+	+++

In addition to the animals included in Table III, each of the two sets of supernatant fluids of experiments 15 and 16 from the three dilutions of filtrate were tested on the same chicken, 0.2 cc. of each being injected intradermally. The results are summarized in Table IV.

TABLE IV

Tumors from Supernatant Fluids

Exp. no.		Chicken muscle	Rabbit muscle
15	Pure Filtrate	++	+++
	1:1 Filtrate	++	++
	1:2 Filtrate	+	+++
16	Pure Filtrate	(4) −	(8) ++++
	1:1 Filtrate	(3) −	(7) +++
	1:2 Filtrate	(2) −	(6) ++
	1:3 Filtrate	(1) +	(5) ++

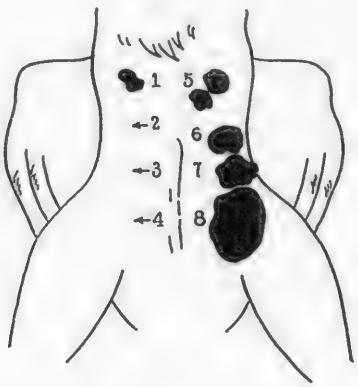

FIG. 1

Fig. 1 gives a schematic representation of the chicken used in Experiment 16, showing the relative sizes of the tumors 16 days after intradermal inocculation.

It appears from these experiments that the amount of tumor agent fixed or inactivated by chicken muscle has a definite quantitative limit.

The difference in activity between the muscle of susceptible animals and of resistant ones is more clearly demonstrated when the filtrates are diluted. The results of the injection of the muscle pulps would suggest that the fixation power of the chicken muscle is stronger than its inactivating power.

Fixation and Inactivation by Desiccated Muscle:—It was desirable to know whether the properties found in the chicken muscle were properties of the living cell in the ground tissue or were reactions between the tumor agent and the tissue constituents. The following experiment shows that dried chicken muscle is able to fix and inactivate the chicken tumor agent almost if not quite as actively as the fresh pulp.

Experiment 17:—The dry muscle used in this experiment was from the same fowl as that used in the fresh state in Experiment 6, when complete inactivation of the filtrate had taken place. The tissues used were dried in a vacuum over sulphuric acid, and then ground to a coarse powder. One gram of each muscle desiccate was mixed with 2.5 gms. of Ringer's solution and after the addition of 2 cc. of fresh tumor filtrate they were kept for two hours at 36°C. and three hours at room temperature. After centrifuging the mixture 1 cc. of each was injected intradermally in normal chickens, and the pulps were given in the same amounts intramuscularly. The activity of the filtrate alone was tested by the injection of 5 cc. intramuscularly. The results are given in Table V.

TABLE V

Tumors from supernatant fluid 1 cc.		Tumors from pulps 1 cc.		Tumors of control from 5 cc. of filtrate alone	
Chicken muscle	Rabbit muscle	Chicken muscle	Rabbit muscle		
+	++++	+	++++	++++	++++

The experiment shows quite clearly the inactivation as well as the fixation of the agent by the dry chicken muscle.

Fixation and Inactivation Experiments with Brain and Liver from Chicken, Rabbit and Pigeon:—The same relative proportions of the organ pulps and fresh tumor filtrate were used as those used in the foregoing experiments with muscles. As indicated by the experiment number, these tests were performed simultaneously with the muscle experiments, the length of contact being the same. The results are given in Tables VI and VII.

TABLE VI

Brain

Exp. no.	Tumors from supernatant fluid 1 cc.		Tumors from pulps 1 cc.		Control tumors from filtrate alone	
	Chicken brain	Rabbit brain	Chicken brain	Rabbit brain	Right side	Left side
7	++ ++	++ ++	++ ++	++ ++	0.5 cc. ++	1 cc. +++
18	+++ ++	+++ ++	+++	+++	5 cc. –	10 cc. –
		Pigeon brain		Pigeon brain		
10	+++	+++	++++ ++++	++++ ++++	0.5 cc. +++ 2 cc. +++	1 cc. +++ 4 cc. ++++
12	– –	– –	+++	+++	1 cc. ++++ 2 cc. –	0.5 cc. +++ 4 cc. –

TABLE VII

Liver

Exp. no.	Tumors from supernatant fluids 1 cc.		Tumors from pulps 1 cc.		Control tumors from filtrate alone	
	Chicken liver	Rabbit liver	Chicken liver	Rabbit liver	Right side	Left side
7	++ + ++	++ + ++	+++ +++ ++	+++ +++ +	0.5 cc. ++ 5 cc. ++++	1 cc. +++ 10 cc. ++++
18	++ +++ ++	++ +++ ++	– –	– –	0.5 cc. – 5 cc. –	1 cc. – 10 cc. –
		Pigeon liver		Pigeon liver		
13	+++ +++ +++	+ +++ +++	++++ ++++	++ ++	0.5 cc. ++++	1 cc. ++++
14	++++ ++ +	++++ ++++ +	+++ +++	+++ ++	0.5 cc. ++	1 cc. ++++

It will be noticed in Experiments 7, 13 and 14 that the simultaneous experiments dealing with muscle gave negative results, due to the excessive activity of the filtrate. Therefore, as far as chicken liver and brain are concerned, these experiments show at least that they are not endowed with a stronger inactivating power than muscle. Experiments 10 and 12 show pronounced fixation with muscle so that a proper control exists for these two experiments, and as the filtrate used in Experiment 19 was weak it is possible that any fixation would have been detected in this experiment.

It seems, therefore, that not only do the liver and brain of rabbits and pigeons fail to show any fixating or inactivating properties for the chicken tumor agent, but that is true as well of the same organs of the chicken.

In addition to the experiments above, two other tissue were tested for the fixating power on the chicken tumor agent, namely, a mouse sarcoma and a non-filterable chicken tumor. In neither tissue was there any indication of fixation while two of the four control tests with chicken muscle showed complete fixation of the agent.

Attempts to Release the Agent from the Inactive Muscle-Filtrate Mixtures:—As noted by Marie and Tiffeneau (7), desiccation of the inactive mixture of brain and tetanus toxin releases the toxin. The technique was used in an attempt to release the tumor agent from the susceptible muscle.

Experiment 19:—The pulps from Experiment 11 were desiccated in a vacuum over sulphuric acid and four days later injected in an amount equivalent to 4 cc. of the fresh pulp.

The results obtained 15 and 30 days after the injection are given in Table VIII.

TABLE VIII

	Tumor from chicken musc.	Tumor from pigeon musc.		Tumor from chicken musc.	Tumor from pigeon musc.
15 days	–	+++	30 days	++	++++

It seems that the agent already fixed and inactivated is not released by desiccation. In fact, in this particular experiment, the injection

of dried pulps showed a greater contrast between the actions of the two muscle mixtures than did the fresh mixture as shown in Experiment 11.

General Comparison of Results with Muscle from Susceptible and Non-susceptible Animals:—If all the tests with muscle of susceptible chickens used in the above experiments be compared with the results with pigeon and rabbit muscle, striking contrast between the groups will be noted. (Table IX.) In the 47 tests with chicken muscle 76% showed unmistakable evidence of some fixation of the tumor agent, and in 34% this was complete. On the other hand, with the 43 tests with muscle from non-susceptible animals there were no instances of fixation. The figures for inactivation, while not based on so many tests show a strong contrast between chicken muscle on the one hand and the rabbit and pigeon muscle on the other.

TABLE IX

Results of Injection of Supernatant Fluids

Muscle from	Number chickens inoculated	Total fixation	Partial fixation	No fixation	Percentage tot. and part fixation	Percentage total fixation
Rabbit	19	0	0	19	0.0	0.0
Pigeon	24	0	0	24	0.0	0.0
Chicken	47	16	20	11	76.7	34.4

Results of Injection of Pulps

Muscle from	Number chickens inoculated	Total inactiv.	Partial inactiv.	No inactiv.	Percentage tot. and part inactiv.	Percentage total inactivation
Rabbit	12	0	1	11	8.3	0.0
Pigeon	12	0	1	11	8.3	0.0
Chicken	24	2	9	13	45.8	8.3

DISCUSSION

The evidence developed in this study seems to indicate that the agent of Chicken Tumor I is bound *in vitro* by muscle tissue from susceptible fowls while the muscle and the tissues from non-susceptible animals such as rabbit and pigeon are devoid of any such action. As

far as the present observation goes, even such non-mesenchymatous organs as the brain and liver of the susceptible fowl show no affinity for the tumor agent *in vitro*. It is of interest in this connection to note the early observations of Murphy and Rous (8) who showed that when tumor filtrate was injected into the chick embryo, tumors developed only in the mesodermal layers of the embryonic membranes.

The question naturally arises as to whether it is solely the muscle cell, the sarcolemma or both which are responsible for the binding action of the pulp. Although this point does not modify the essential nature of the phenomenon, the supposition that a transformation of the differentiated muscle cell under the action of the agent into a malignant cell does not seem theoretically unreasonable.

The interaction between the muscle of susceptible fowls and the tumor agent resembles in its specificity the binding of the antibodies by antigens, bacteriophage by the sensitive bacteria and the enzyme by the specific substratum. The muscle tumor agent combination seems to be rather stronger than some of the examples quoted, as desiccation does not release the activity. The delicacy of the tumor agent prevents more extensive attempts to dissociate the combination with muscles. It is true that certain viruses also have a high degree of specificity in animals and plants and share with the tumor agent the property of requiring living matter for their multiplication. However, it has been demonstrated (9) that at least one typical virus, the vaccine virus, is not bound or inactivated by contact with sensitive tissue from susceptible animals. This is possibly a fundamental difference between the behavior of the chicken tumor agent and the filterable agents of the virus group.

CONCLUSIONS

Ground muscle from susceptible chickens fixes in vitro in a proportion of instances the agent of the filterable Chicken Tumor I, and in a lesser degree inactivates it, whereas the muscle from resistant animals such as rabbit and pigeon, is without effect. It is shown that the power of fixation of the chicken muscle is far greater than its inactivating properties.

Brain and liver from chicken, rabbit and pigeon seem devoid of any action on the agent.

The desiccated chicken muscle tissue shares the properties of the fresh organ; and the process of desiccation does not release the agent from the inactive or slightly active mixture of fresh muscle and filtrate.

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THE EFFECT OF EXTRACTS OF CERTAIN ORGANS FROM NORMAL AND IMMUNIZED ANIMALS ON THE INFECTING POWER OF VACCINE VIRUS

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PLATES 14 AND 15

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In a previous paper (1) we reported that when the agent of Chicken Tumor I was brought in contact with finely ground muscle from susceptible animals it was bound so that both the supernatant fluid and the muscle tissue became partially or completely inactive when injected into chickens. The muscle of non-susceptible animals such as pigeon and rabbit, or even organs such as liver, brain and kidney of susceptible chickens do not affect the activity of the agent.

The present study was undertaken in order to determine whether a similar effect could be demonstrated for a typical representative of the group of the so-called filter-passing viruses, such as vaccine virus. As laboratory animals are more or less susceptible to vaccine virus, for the refractive group it has been necessary to use artificially immunized rabbits, although it is realized that subsequent work may show that the comparison is unjustified.[1]

The fact that tissues of immunized animals inactivate the virus *in vitro* has already been shown. Thus Levaditi and Nicolau (4) report that vaccine virus and herpes virus were "destroyed" by the brain tissue from immunized animals. Tessier, Gastinel and Reilly (5) using a small number of animals confirmed the above result with herpes virus and further determined that the brain and serum from naturally immune rabbits and non-susceptible dogs are devoid of action on the virus.

[1] A preliminary note on the subject has already been published and some of its points have been duplicated on the staphylococcus (2), (3).

Methods and Materials

Vaccine Virus.—Three different strains of virus have been used, namely, the Noguchi testicular vaccine virus, the Levaditi neurovaccine, and the cow glycerinized dermovirus of the Department of Health, City of New York.[2]

The specimen of testicular virus was prepared by injecting 0.5 cc. of vaccine emulsion mixed with an equal amount of Ringer's solution into both testicles of a rabbit. 5 days later, when the resultant orchitis was at its height, the animal was killed, and the testicles removed aseptically. They were ground thoroughly with sand together with 25 cc. of glycerol and 25 cc. of Ringer's solution. This emulsion was distributed in tubes, covered with a layer of sterile vaseline, and kept in the ice-box.

The specimen of neurovirus was prepared by injecting about 0.2 cc. of a heavy suspension of infected brain tissue into the brain of a rabbit. After 5 days the brain was removed aseptically and treated in the same manner as the testicular virus. Dilutions, usually at 1:50, were made immediately before each experiment with Ringer's solution.

The animals were carefully shaved, so as to avoid any injury of the skin, and generally a test injection was made intracutaneously on one side, the opposite side being used as control in order to avoid the disturbing fact of the individual differences in susceptibility.

Inactivation of Testicular Virus by Immune Brain Extract.—As mentioned above, Levaditi has shown that an extract of the brain of an immune animal neutralized the neurovaccine. This experiment has been repeated, substituting Noguchi's testicular stain for the neurovirus.

The brains of two rabbits immune to the testicular virus were used, controlled by the brains from two normal rabbits. A half of each brain was ground and mixed with 2 to 3 cc. of a 1:10 virus dilution. The mixture was kept for 5 hours at room temperature in one instance and in the other for 3 hours at 37° and then overnight in the ice-box. The mixtures were then centrifuged and the supernatant fluids and pulps injected separately into normal rabbits. The results are shown in Table I.

TABLE I

Exper.	Lesions produced by supernat. fluids		Lesions produced by pulps	
	Immune brain	Normal brain	Immune brain	Normal brain
I	0.5 cc. ±	+	0.1 cc. −	+
	0.5 cc. ±	+	0.1 cc. ±	±
	0.5 cc. −	+	0.1 cc. ++	++++
II	0.5 cc. −	±	0.1 cc. +	+
	0.5 cc. −	−	0.1 cc. +	+

[2] We wish to acknowledge our indebtedness to Dr. T. M. Rivers for supplying the original strains of testicle and neurovirus.

Inactivation of Testicular Virus by Immune Testicle Tissue.—The same experiment was repeated using testicle instead of brain tissues.

Immune rabbits from 1 to 3 months after the immunizing injection of testicle or neurovirus were used. The testicles were removed and ground without sand, and measured amounts of the resultant pulps were mixed with equal volumes of 1:50 testicle virus dilution. The length of contact was 3 hours at room temperature in one instance and from 1 to 3 hours at room temperature and overnight in the ice-box for the rest of the experiments. The mixtures were then centrifuged and the supernatant fluids injected into normal rabbits. The same procedures were carried out with normal testicles in the control experiments.

The results are summarized in Table II.

TABLE II

Exp. No.	Lesions by supernat. fluids from:		Lesion by pulps from:	
	Immune testicle	Normal testicle	Immune	Normal
3	0.2 cc. −	+++	0.2 cc. −	+
	0.5 cc. ±	++++		
4	0.2 cc. ±	+++	0.1 cc. −	+++
	0.5 cc. ±	+++	0.1 cc. −	+++
5	0.2 cc. −	+++	Scarification −	+
	Scarification −	++		
6	0.5 cc. −	++	No test	No test
7	0.2 cc. +	++	0.1 cc. +	++
8	0.3 cc. −	++	0.1 cc. −	++
9	0.5 cc. −	+++	0.1 cc. −	++
10	0.5 cc. −	+++	0.2 cc. −	±
11	0.5 cc. −	+++	0.2 cc. +	+++
	0.5 cc. −	+++	0.2 cc. +	++

It is evident from these tests that the brain and the testicle from immune animals inactivate vaccine virus, the latter tissue being the

more active. The inactivation takes place quickly, as a contact of 3 hours reduced the activity of the virus dilution as completely as a longer contact.[3]

Perfusion Experiments.—It is known that the serum from an immune animal exhibits so-called viricidal action on vaccine virus. Hence the results obtained so far might be ascribed to the inactivating power of the blood contained in the tissue and not the tissues themselves. To check this point the following experiments were performed.

A 3 months immune rabbit was perfused under anesthesia with Ringer's solution and sodium citrate through the abdominal aorta and the vena cava until the testicles became perfectly white. The same procedure was carried out on a normal rabbit and the above experiments were repeated with this material. The mixture of testicle and virus was kept at room temperature for 3 hours and then overnight in the ice-box. A similar experiment was carried out with neurovirus. The results are given in Table III.

TABLE III

Virus	Lesion by supernatant fluids		Lesion by pulps	
	Immune testicle	Normal testicle	Immune testicle	Normal testicle
Testicle	1 cc. −	++++	0.1 cc. ±	++
Neuro-	1 cc. +	Very extensive lesion	0.1 cc. +	++++

It appears that the lesions from immune animals possess an inactivating power as well as the serum.

Speed of Reaction between Vaccine Virus and Immune Organs.—A

[3] The question of whether or not the testicle tissue from suscepticle animals fixes the virus was answered by a set of experiments in which normal rabbits were injected with testicle emulsions immediately after the mixtures were made and again after some hours of contact. As controls the same amount of virus dilutions as those contained in the mixtures was also injected at the same time. As there was no difference in the extent of the lesions produced it may be concluded that there was no fixation of the virus by normal testicle tissue. That there was a mechanical retention or adsorption of the virus by the tissue was shown by the fact that washing of the tissue after contact did not materially reduce the activity of the material when injected into animals.

further attempt was made to determine the speed of reaction and its further progress during the time of contact.

3 cc. of ground testicle tissue from a 35 day immune rabbit was mixed with an equal volume of a 1:50 testicular vaccine dilution. Mixtures were immediately centrifuged and a sample of the supernatant fluids injected intrademally into a normal rabbit. The remaining fluid and tissue were mixed and allowed to remain in contact for 5 hours at room temperature and then for 17 hours in the ice-box. The mixture was again centrifuged and another sample of the supernatant fluid was tested on the same rabbit. A similar experiment was carried out with neurovirus. The results are given in Table IV.

TABLE IV

Virus	Lesions produced by supernatant fluids immediately after mixing		Lesion by supernatant fluids and pulps after 22 hours of contact	
	Immune testicle	Normal testicle	Immune testicle	Normal testicle
Testicular	0.5 cc. −	+++	0.25 cc. + 0.50 cc. + 0.10 cc. ±	+++ ++++ +++
Neuro-	0.5 cc. +	Very large lesion	0.25 cc. + 0.50 cc. +	++++ ++++

These tests indicate that the inactivation of the virus by the testicle from immune animals is accomplished very quickly and is not notably increased by further contact.

The possibility that the virus was inactivated but not destroyed by the contact with tissues from immune animals was tested. The mixtures subjected to tryptic digestion, desiccation or treatment with solutions of different pH failed to release the virus in active form.

Enhancement of Neurovirus by Normal Testicular Extract.—It will be noted that in the above experiment the neurovirus was inactivated by the testicular virus. On the other hand, the normal testicular extracts, which are devoid of appreciable effect on the testicular strain, gave evidence of an enormous augmentation in the skin lesions pro-

duced by the neurovirus. This observation was confirmed by the experiments which are summarized in Table V.

TABLE V

Lesions produced by the supernatant fluid from:		Lesions produced by the pulps from:	
Immune testicle	Normal testicle	Immune testicle	Normal testicle
1. 0.2 cc. +	Very large lesion	0.2 cc. +	Very large lesion
2. 0.2 cc. ++	Very large lesion	0.2 cc. +	++++
3. 0.2 cc. +	Very large lesion	0.2 cc. +	++

The lesions were much more hemorrhagic and necrotic and spread over a much greater area than those produced by the virus alone. The two rabbits injected with the supernatant fluid from the mixture of normal testicular extract plus neurovirus became very sick, lost considerable weight, and died 6 and 7 days respectively after the injection. One of the rabbits injected with the pulp showed also a large lesion and became sick but eventually recovered. The other rabbits showed the usual vaccinal eruption.

Detailed Study of the Enhancement of the Neurovaccine Virus by Testicle Extracts.—The nature of the lesions obtained in the foregoing set of experiments warranted a further and careful study of the power shown by normal testicle extracts in enhancing to such a high degree the infecting power of the Levaditi neurovirus.

The technique at first was the same as in the foregoing experiments, and pulps as well as supernatant fluids were injected. Later on the procedure was simplified by grinding the testicle together with its volume of Ringer's solution and centrifuging immediately. Only the cloudy supernatant fluid was used. About 0.5 cc. of this was mixed with 0.25 cc. of a 1:50 dilution of infected tissue emulsion immediately before the injection.

With the supernatant fluid the enhancement has been observed in practically all of the 80 tests carried out. In 2 or 3 instances where the enhancement was doubtful or negative, the effect was traced to the use of a feeble virus or atrophic testicles. The injection of the pulps in the amounts used did not give constant results, as in 5 out of 11 instances there was no enhancement, and in 2 cases only doubtful enhancement.

Only in a few instances did the virus-testicle mixture give an earlier lesion than the virus alone. Generally on the 2nd or 3rd day evidence of the infection became detectable on both sides, but the lesion was very much less extensive in the control side. The lesions produced by the neurovirus alone generally reached their maximum spread by the 4th or 5th day and presented the usual picture of a more or less hemorrhagic localized eruption. On the other hand the lesions produced by the virus plus testicle extract generally continued to spread till the 6th or 7th day, by which time they had extended throughout the flank and even over the abdomen. The skin appeared thick and very red or violaceous in color, and it was often covered with blisters. Areas of necrosis often appeared in the middle of the lesion and sometimes the whole of the skin area was necrosed. There was generally a marked oedema in the neighbouring regions.

General symptoms in the rabbits injected intradermally with the testicle-neurovirus mixture became pronounced. The temperature reached 105°F. and sometimes 106° and 107°. In most cases hypothermia followed in the days immediately before death. There was considerable loss in weight—sometimes more than 600 gm.—and most of the animals developed severe diarrhea and signs of pulmonary disease. Conjunctivitis was observed in a few instances.

Death occurred in about 25 per cent of cases. The most striking findings at postmortem examination were a double hemorrhagic lobar pneumonia with sometimes widespread pulmonary abscesses and severe glomerulo-nephritis. Lymph nodes in the vicinity of the lesion were enlarged and congested and the testicles were sometimes congested. The details of the histological lesions of this generalized vaccinal infection will be reported in a later paper. Typical alterations were found in the ovaries, tesicles, suprarenals, lungs, etc., and vaccine virus was easily recovered from these and other organs independently of its presence in the blood.

In other animals purposely killed at the height of the disease, more or less pronounced lesions were also found in the lungs. The general clinical and histo-pathological picture of disease caused by the vaccine virus plus testicle extract is that of the usual infection by the virus alone, but extraordinarily enhanced.

The Influence of Other Organ Extracts on Neurovirus Infection.—It was obviously interesting to know how organs other than testicle behave when injected along with neurovirus.

The same technique was employed, the supernatant fluids only being used. The mixture of virus and tissue extracts or serum was made immediately before injection, in the ratio of 0.25 cc. of a 1:50 dilution of the virus emulsion to 0.50 cc. of the organ or tissue extract.

The results are summarized in Table VI.

TABLE VI

Organ extract injected with neurovirus	No. of tests	No. enhanced	No. unmodified	No. decreased	No. suppressed
Testicle	80	78	2	0	0
Kidney	6	6	0	0	0
Skin	2	2	0	0	0
Suprarenal	4	0	4	0	0
Blood (whole)	3	0	1	2	0
Serum	10	0	5	5	0
Bone marrow	8	0	5	3	0
Lymph nodes	2	0	2	0	0
Spleen	24	0	8	14	2

In addition to the above, one test each was made with several other tissues. The indications from these are that liver, brain and placenta enhance the infection, while muscle, retina and the whole embryo are without effect.

From the study of the results we are able to classify the organs or tissues as regards their influence on vaccinal infection as follows: (1) organs that always enhance, such as testicle, kidney, etc.; (2) organs which neither enhance nor interfere, as, probably, muscle, suprarenal, etc.; (3) organs which never enhance and very often interfere with and even suppress the infecting power of the neurovirus, such as spleen, blood, etc.

Among the organs endowed with an enhancing power the testicle is by far the strongest, whereas the spleen seems most active among the organs which interfere with the infection. It is interesting to note that the inhibiting power of the spleen is quickly lost by dilution, the pulps being the most effective, whereas the enhancing power of the testicle, as will be seen later, is unaffected by high dilutions.

Enhancement of the Dermovaccine Virus by Testicle Extracts.—In view of the fact that the enhancement of the vaccine virus in the skin was positive with the neuro- and negative with the Noguchi testicular virus, it was desirable to determine whether the testicular extract was capable of enhancing the activity of the usual cow dermovirus.

Rabbit testicle extract was prepared in the usual way, and 0.5 cc. was injected intradermally together with the entire content of a tube of dermovirus (a human dose) into the left side of two rabbits. The right side was injected with the virus

alone with Ringer's solution. The left side showed lesions 5 times larger than the right side, but these lesions were milder than those caused by the neurovirus or the testicle virus.

Enhancement of the Noguchi Testicle Virus.—That the feebler activity of the Noguchi testicle virus is not responsible for its lack of activation by testicle extract is clearly shown by the foregoing experiment where a still feebler virus, the cow virus, is definitely enhanced by the same extract.

Kidney extracts proved to be effective in one experiment in enhancing slightly but definitely the testicle virus infection in the skin. In another experiment testicle virus was injected into the brain of a rabbit which was killed 5 days afterwards, and this brain, used as neurovirus, proved to be definitely enhanced by testicle extract. The lesions obtained were the usual mild lesions produced by the testicle virus. On the other hand, the neurovirus after growth in the testicle lost none of its virulence and was still actively enhanced by testicle extracts when injected intradermally. It is therefore obvious that enhancement of testicle virus by organ extracts is possible under certain conditions.

Enhancement of Neuro- and Testicle Virus by Testicle and Kidney Extracts when Injected into the Testicles.—These experiments were performed in order to determine the effect of the enhancement in organs other than skin, namely, testicles.

The general procedure in preparing the material does not differ from that used in the skin experiments. The right testicle was used for injections and the left used for comparison. The results of 4 experiments on 16 rabbits are summarized in Table VII.

TABLE VII

Lesion produced by:			Lesion produced by		
	Testicle virus plus organ ext.	Testicle virus plus Ringer's		Neurovirus plus organ ext.	Neurovirus plus Ringer's
Testicle.....	+	++	Testicle.....	+++	+++
Kidney.....	+++	−[4]	Testicle.....	++++	+++
Testicle.....	+++		Testicle.....	++++	
Kidney.....	+++	−	Kidney.....	±	+
Kidney.....	+		Kidney.....	++++	

[4] This minus sign does not show complete absence of orchitis but simply the complete absence of any gross inflammation. That infection had taken place is shown by the fact that an active virus may be recovered.

From the study of this table we can draw the conclusions that, in spite of some irregularity of both neuro- and testicle virus in giving rise to definite orchitis in rabbits, both can be enhanced by either testicle or kidney extract when injected into rabbit testicles.

Experiments on the Nature of the Enhancement.—The enhancement is not species specific, as the tests show that rabbits injected with neurovirus plus rat or guinea pig testicle extract, prepared and used as the rabbit extract, exhibit typical enhanced lesions. All animals injected became sick and some died on about the 8th day, with the usual general signs especially in lung and kidney. The age of the animal from which the organs for the extracts are taken, as well as the age of the injected animal, has no influence on the phenomenon of enhancement. Animals solidly immune to both neuro- and testicle virus are as resistant to infections by the virus plus testicle extract as by the virus alone. Slightly autolyzed extracts are endowed with the same power as extracts recently obtained.

The virus does not seem to be modified in its virulence, for strains secured from the enhanced lesions are no more highly infective than those secured from the ordinary lesion. That, at least, the action of the extracts is directed toward the cell is shown by the occasional observation that some rabbits having pronounced lesions from pure neurovirus showed a typical vaccinal eruption in spots injected only with testicle extract. This suggested an experiment in which the shaved skin of a rabbit received in three different spots testicle extract,[5] and at the same time 1 cc. of neurovirus diluted with 20 cc. of Ringer's solution was injected into the ear vein. Typical lesions developed at the three injected spots and the animal died on the 4th day, showing that the blood virus localized only in the sensitized spots. After the injection of testicle extract the cells remained sensitized for a certain length of time. This was shown by an experiment in which 5 rabbits were injected intradermaly with 0.5 cc. of the extract while in the course of the following days 0.25 cc. of a 1:50 dilution of the virus was injected in the same spots. Another spot on the skin was injected with the virus alone in each case as a control. Enhanced lesions were

[5] The extract used in this experiment was kept 24 hours at room temperature and 24 more in the ice-box. Two similar experiments dealing with testicle virus gave negative results.

obtained up to the 3rd day, whereas later the lesions were not different from those obtained in the control spots, or the enhancement was doubtful. The virus used was of moderate strength, so that perhaps a longer sensitization would have been observed with a more active virus.

Preliminary Experiments on the Physical and Chemical Nature of the Enhancing Substance.—As the result of a number of preliminary experiments some of the properties of the enhancing substance have been determined. Dilutions of the supernatant fluids of testicle extracts, as high as 1:160 are almost as active in their enhancing power as the full strength extracts. Berkefeld filtrates of the extracts contain the enhancing substance in considerable amounts. Exposure to 100°C. for 3 minutes completely destroys the activity of the extracts. The precipitate resulting from the acidification of a Berkefeld filtrate of testicle extract contains practically all of the enhancing substance. This precipitate gives uniformly a strong Feulgen reaction, indicating a predominance of nucleoprotein elements.

DISCUSSION

The experiments reported here show that the neuro-, testicular and cow strains of vaccine virus are not only uninjured by contact *in vitro* with sensitive tissues from susceptible animals, but their infectivity is enhanced to an extraordinary degree by such contact. This finding is in strong contrast to the results obtained when a chicken tumor agent is subjected to similar treatment. The fact that one agent is either bound or inactivated by the constituents of the more susceptible tissue while another has its activity greatly enhanced suggests a fundamental difference in the mechanism involved in the action of the two and tends to separate them into different classes. It is known that certain enzymes form a union with the specific substance on which they act and this is supposed to be true in general for the enzyme-like agents. Whether or not the virus group is uniformly unaffected by contact *in vitro* with tissues of susceptible animals, or may have their activity enhanced by such tissues, remains to be determined. In the instance of the vaccine virus the evidence is conclusive.

The result of contact of vaccine virus with tissue extracts from immune animals and the chicken tumor agent with tissues from susceptible fowls is the same, namely, inactivation in both instances. The fact that vaccine virus is inactivated particularly by the sera from immune animals is well known and is generally considered to be the result of a real destruction of the virus. But the fact that the reaction between the virus and the immune tissue extract reaches its maximum almost immediately after the contact is effected and does not progress, and furthermore, because of the similarity between this effect and the inactivation of the chicken tumor agent by sensitive tissues, seemed to indicate that the action was not a destruction of the virus. The recent work of Andrews (6) has shown that the active virus may be recovered after contact with immune serum even when the serum is used in great excess. Furthermore Long and Olitsky (7) have shown that active vaccine virus may be recovered by cataphoresis from the testicle of immune animals many months after the disappearance of all active lesions. Hence it seems that the virus is not necessarily destroyed by the immune substance.

The most active of the tissues in the augmentation of the infectivity of the vaccine virus is the testicle. It is of interest to note that this organ is not only the most sensitive tissue to direct inoculation but is with the ovary the site where, in absence of previous irritation, the vaccine virus localizes most abundantly after intravenous inoculation.

The mechanism of the enhancement is not clear. The fact that the virus injected into an area of skin some hours or days after the tissue extract has been injected into the same area, results in an enhanced lesion, suggests that the effect of the extract is on the host cells, rendering them more susceptible to the virus, rather than on the virus direct. This interpretation is further strengthened by the observation that virus injected intravenously localizes most readily in areas of skin previously injected with testicle extract, and the lesions resulting are very extensive. It may well be that the extracts act primarily as a stimulus to cell division, thus increasing the number of young cells which are supposed to be more susceptible to the virus effect than older cells.

Aside from the organs or tissues whose extracts enhance the activity of vaccine virus there are others which frankly interfere with the infec-

tion. Among these latter the spleen pulp shows greatest inhibiting effects. In this connection evidence associating lymphoid reaction with the inhibition of tissue growth is recalled. But regardless of the explanation of the phenomenon described it seems that a basic difference has been established between the behavior of the filterable agent of the chicken tumor and a typical member of the so-called filter-passing viruses.

SUMMARY

Brain and testicle tissue from immune rabbits brought in contact with the Levaditi or Noguchi strains of vaccine virus will fix or inactivate the virus. Extracts of normal testicles from susceptible animals enhance to an extraordinary degree the infectivity of both the neuro- and dermal strains of vaccine virus. The Noguchi virus is not affected by testicle extracts when injected into the skin, but kidney extract has a definite enhancing power on the strain when injected into either skin or testicle.

The effect of tissue extracts seems to be on the cells of the host rather than on the virus. This is indicated by the fact that virus injected intravenously localizes most readily in an area of skin previously injected with testicle extract. Furthermore an enhanced lesion results if the virus is injected into an area as long as 3 days after the area has been injected with testicle extract.

The augmenting substance of the tissue extracts is little affected by high dilutions, passes through a Berkefeld V candle and is carried down with the proteins precipitated by weak acids.

Rabbits with enhanced lesions show general symptoms and about 25 per cent die with generalized vaccinia.

Kidney, and probably skin, brain and liver extracts possess enhancing properties, but to a much less degree than the testicle. On the other hand, spleen, blood and probably lymph nodes and bone marrow not only fail to produce enhancement, but actually restrain or even suppress entirely the vaccinal skin infection.

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EXPLANATION OF PLATES

PLATE 14

FIG. 1. (Rabbit 160, right side). Lesion produced by the intradermal injection 4 days before of 0.25 cc. of a 1:50 dilution of neurovirus plus 0.50 cc. of the precipitate obtained by the addition of acid to the Berkefeld and concentrated testicle extract.

FIG. 2. (Rabbit 160, left side). Control. Lesion produced by 0.25 cc. of the virus dilution plus 0.50 cc. of Ringer's. Note in the lower abdomen the oedema from the right side lesion.

FIG. 3. (Rabbit 163, right side).

1. Lesion produced by the injection 5 days before of 0.25 cc. of a 1:50 dilution of neurovirus plus 0.50 cc. of testicle extract.

2. Lesion produced by 0.25 cc. of the virus dilution plus 0.45 cc. of spleen extract.

FIG. 4. (Rabbit 163, left side). Control. Lesion produced by 0.25 cc. of a 1:50 dilution of virus plus 0.50 cc. of Ringer's.

PLATE 15

FIG. 5. (Rabbit 97, right side).

1. Lesion produced by the injection 8 days before of 0.25 cc. of a 1:50 virus dilution plus 0.50 cc. of testicle extract.

2. Lesion produced by the injection 8 days before of 0.25 cc. of a 1:100 virus dilution plus 0.50 cc. of testicle extract.

3. Lesion produced by 0.25 cc. of a 1:100 dilution of neurovirus plus 0.50 cc. of kidney extract.

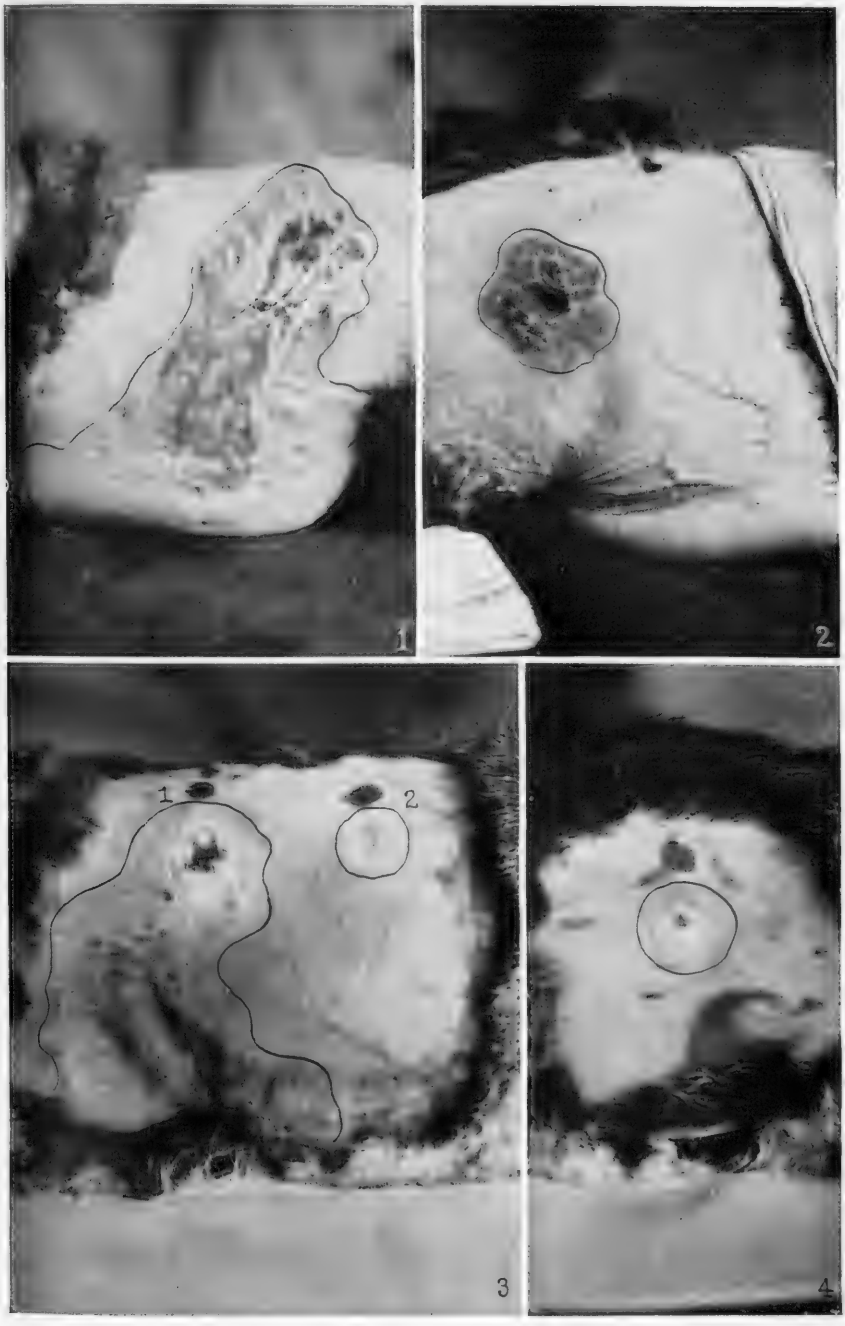
FIG. 6. (Rabbit 97, left side). Control.

1. Lesion produced by the injection 8 days before of 0.25 cc. of a 1:50 neurovirus dilution plus 0.50 cc. of Ringer's.

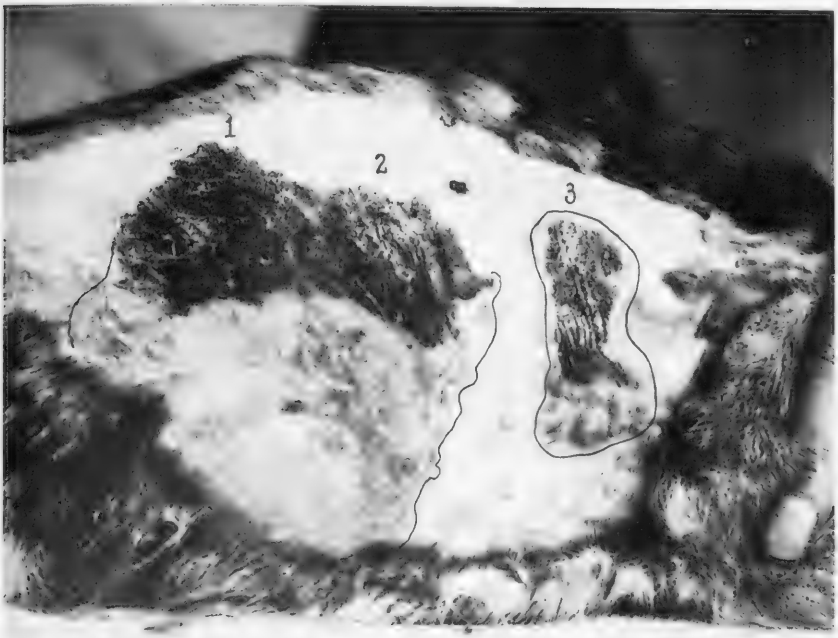
2. Spot injected with 0.50 cc. of kidney extract alone.

3. Spot injected with 0.50 cc. of testicle extract alone. Note the secondary localization of the virus.

(The rabbit died 2 days after the photographs were taken.)



(Duran-Reynals: Infecting power of vaccine virus)



5

6

(Duran-Reynals: Infecting power of vaccine virus)

A STUDY OF THE GENERALIZATION OF VACCINE VIRUS FROM ENHANCED SKIN LESIONS

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PLATES 16 TO 18

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One of us has shown in a previous paper (1) that extracts from normal testicles are endowed with the power of enhancing to an extraordinary degree the lesions produced by vaccine virus. Extracts from kidney, liver, skin, brain and placenta share this property only to a certain extent. Extracts from suprarenal, retina, muscle and ovary do not modify the vaccinal infection, whereas blood, spleen and bone marrow extracts frankly interfere with and even suppress the activity of the virus.

Rabbits injected intradermally with mixtures of neurovirus and testicle extract become ill, develop a very high temperature, lose considerable weight, and approximately 25 per cent of the animals die from the disease. The present study is chiefly concerned with the histological characteristics of the wide-spread, typical vaccina, together with the non-specific lesions found at autopsy, and with the bearing of these findings on the much discussed question of the tissue affinities of the vaccine virus.

Calmette and Guerin (2) found that when vaccine virus was injected intravenously, it localized selectively in the shaved areas of the skin, whereas no localization was observed in mesodermic organs. This phenomenon was also observed by Camus (3), who found moreover that the virus localized in the buccal mucous membrane, genital organs and in cutaneous *naevi*. It was likewise noted by Levaditi and Nicolau, Rivers and Tillett, Noguchi and others (4, 5). Borrel and Burnet (6, 7) introduced the term *épithélioses* for a group of diseases produced by filter-passing viruses; viz., vaccinia, variola, fowl-pox, sheep-pox and molluscum contagiosum, all of which were said to attack epithelium almost exclusively. Lipschütz (8) promulgated the theory that the tissue localization of a virus is

conditioned by so-called specific affinities. A similar hypothesis was sustained by Menze (9). The doctrine of the *ectodermoses neurotropes*, however, has been defended mainly by Levaditi and his coworkers. Vaccine virus is regarded as a typical member of this group, showing a marked affinity for the internal segment of the ectoderm (neuraxis) and a more or less pronounced affinity for the external segment of the same ectoderm. The mesoderm and blood, therefore, would be considered wholly non-susceptible, and only certain entodermic organs would be involved.

In 12 experiments performed by Levaditi and Nicolau (10) vaccine virus was injected into rabbits, usually by the intravenous route. These rabbits were killed in from 6 to 10 days. The virus did not localize, or else was poorly localized, in mesodermic organs, whereas it did localize in the mammary glands, adrenals, tongue and buccal mucosa, and likewise in the skin and brain, but only after irritation. It localized abundantly in the lungs and moderately in the liver and the submaxillary salivary glands. Histological lesions, infiltrative or necrosing, were found in the mammary glands, and on two occasions in the lungs and liver. They were always absent in mesodermic organs. Of all the structures studied by Levaditi and Nicolau, the testes and ovaries were regarded as the most sensitive to vaccine virus because the virus could readily be recovered from them and because, at histological examination, necrotic lesions, interstitial in distribution, were consistently found.

The susceptibility of the testicle had already been shown by the work of Noguchi (11), Ohtawara (12), Rivers and Tillett (loc. cit.) and others, who found that the testis was the most suitable organ in which to demonstrate very small amounts of virus. Moreover, Parker (13), Merwin and Schemerling (14), and Eagles and McClean (15) reported data showing that testicular tissue is an excellent substrate for the *in vitro* cultivation of vaccine virus.

The concept of *ectodermoses neurotropes* was enunciated in 1921 (16). Since then there has been a reaction against it, since later experimental work has failed to bring supporting evidence. Thus, Blanc and Caminipetros (17), Chaumier (18), Walthart (19) and Ledingham and McClean (20) succeeded in implanting and passing vaccine virus in pure mesodermic tissues, such as muscle, kidney, lymphatic tissue and derma (corium) without any loss of activity. Moreover Ohtawara (loc. cit.) and Gildemeister and Heuer (21), after intratesticular inoculation, withdrew the virus from the blood as late as the 15th day after the injection. Recently Wilson and Smith (22) by the use of a special technique of fractionation, found the virus adherent to the white blood cells up to 8 days after injection. Vaccine virus has been successfully cultured *in vitro* in a kidney medium by H. B. and M. C. Maitland (23).

Watanabe (24), in a small series of animals injected intravenously, found that the virus localized especially in the dermis without involvement of the epithelial cells; it was likewise found in the liver and spleen.

A very extensive study of the subject has been made by Douglas, Smith and Price (25), who, after intravenous injections in a particularly sensitive strain of

rabbits, noted that the virus produced macroscopic specific lesions (pocks) in the following organs, arranged in order of their frequency of involvement: first, lung, skin and mucosa, spleen, and liver; secondly, genital organs and adrenals, both with the same percentage incidence of lesions. After intradermal and cerebral inoculation some generalization was detected, especially in the lungs, liver, skin and spleen. The tissues were tested by sub-inoculation for infectivity and it was found that during the first two weeks the virus was abundant in the lungs, spleen, bone marrow and genital organs; after this time, however, the skin, tongue, adrenals, ovaries and testicles seemed the most prone to harbor the virus. These authors' conclusion was that vaccine virus "possesses varying degrees of affinity for different tissues and general statements of tissue susceptibility to vaccinia virus should however be received with caution."

Ledingham (25) believes that the reticulo-endothelial system is in the main affected and responsible for the vaccinal infection, the epithelial lesions being secondary to the attack on the mesodermal derivatives. This conclusion was reached both by the histological study of the experimental lesion and by the fact that a blockade of the system in the skin with India ink frankly interfered with the infection. Several authors have already regarded the variolous pocks in the skin as being purely inflammatory. MacCallum and Moody (27) state that in alastrim the corium is involved before the epithelium.

The literature concerning the pathological anatomy in variola and allied diseases is most extensive. A complete account of the work previous to 1904 will be found in the memoirs of Councilman, Magrath and Brinkerhoff and their associates (28), but most of these papers are concerned with the parasitic nature of the virus and deal chiefly with skin and corneal lesions where Guarnieri bodies are easily found. Nevertheless the above-mentioned authors give a very complete description of the generalized lesions in variola, noting in the testes specific "anemic focal necrosis," and furthermore degeneration apparently not truly anemic in origin, but "due to the action of toxins," in the blood-forming cells of the bone marrow which constitute for them lesions almost pathognomic of variola but nevertheless devoid of "parasites." Chiari described these bone marrow lesions, applying the term "osteomyelitis variolosa." Councilman and his associates likewise noted the constant occurrence of more or less acute diffuse degenerative changes in liver, kidney, suprarenals and testes.

The most common lesion found in the lung and one which was very rarely absent was bronchitis, usually combined with more or less extensive bronchopneumonia even when this was not grossly demonstrable.

As early as 1886 Chiari (29) found in the testicles of 15 variolous children and in 85 per cent of adults with smallpox, typical foci with a central area of necrosis surrounded by an area of cellular infiltration. He regarded these lesions as being due to the direct action of the virus. Similar necrotic specific changes in the bone marrow and testes have been found by MacCallum (31) in alastrim. MacCallum mentions the intense cloudy swelling and focal necrosis in the liver, and the exten-

sive degenerative changes in the kidneys. In most cases there was an acute bronchitis, and in many there occurred a rather severe lobar pneumonia.

Material and Methods

The rabbits used were mostly of the common gray variety; some, however, were of the albino type. No essential difference between them could be detected. The age of the animals appeared of no significance.

The animals studied were grouped and treated as follows:—

A) Twenty-one rabbits were injected intracutaneously with neurovirus plus testicle extract. Twelve of these died as a result of the infection. The others were killed when in a more or less serious general condition. All of them were autopsied during the course of extensive specific lesions in the skin, the area involved often occupying the whole flank and abdomen.

B) Eight rabbits were injected intravenously with either 1 or 2 cc. of neurovirus diluted to 1:10 or 1:20, alone or mixed with rabbit testicle extract. Two of the animals died; the others were killed.[1]

C) Two rabbits were injected in the testis, two more in the brain and one directly in the liver.

The disease resulting from the injection of neurovirus plus testicle extract when inoculated together into the skin has already been described (1).

Gross Lesions

At the postmortem examination of the animals dying from vaccinia the following changes were observed:

A markedly edematous zone of hemorrhagic character widely surrounded the borders of the vaccinial lesion in the skin. The lymph nodes of the drainage area were very much congested and enlarged. There was marked congestion of the splanchnic organs—kidneys especially; the liver was enlarged. The peritoneal cavity frequently contained a few cubic centimeters of viscid hemorrhagic fluid. The muscles of the abdominal wall occasionally showed discrete punctate hemorrhagic areas. There was uniformly congestion of the testes with some fluid in the tunica vaginalis. The most obvious gross changes were found in the lungs where

[1] The virus was prepared as described in other papers. This particular sample was of a uniform activity, giving positive takes in the skin at a dilution as high as 1:2.000.

the lesions consisted of foci of congestion and patches of lobular pneumonia. We are fully aware of the doubtful significance of such lesions in the rabbit lung. The pleurae and pericardium were occasionally involved, the serous cavities being distended by fluid.

In the animals killed the intensity of the lesions bore a comparable relationship to the gravity of the clinical condition.

Pocks in internal organs, as described by Douglas, Smith and Price, were never encountered. Only in the suprarenals and ovaries the existance of clean-cut and more or less hemorrhagic foci with superficial elevation of the gland surface enabled one to make the diagnosis of vaccinal infection macroscopically. The testicles likewise showed these foci, but less clearly. Skin generalization was observed but very rarely. In more than 200 rabbits with simple or enhanced cutaneous or testicular lesions, typical pocks were encountered with some regularity, although in small numbers, in the tongue and lips alone.

It must be kept in mind that we used the same strain of virus that Douglas, Smith and Price used, so that evidently idiosyncrasy in the rabbits is of paramount importance as regards the generalization and character of lesions produced by the neurovirus.

Microscopical Findings

Material was fixed in Zenker's fluid and thin sections—some 4 micra in thickness—were stained in Mallory's eosin-methylene blue, phloxine-methylene blue, or by Wolbach's modification of the Giemsa stain. Lesions regarded as specifically vaccinal were found by histological examination in lung, liver, spleen, lymph nodes, bone marrow, adrenal, ovary, and testis; one doubtfully specific lesion was encountered in the kidney. Changes considered as accessory and not definitely specific were found in spleen, lymph nodes and kidneys. In the specific visceral lesions nothing of the nature of Guarnieri bodies were found after long search, save perhaps in the ovary, where, in two animals, structures were present in Graafian follicle cells adjacent to an active vaccinal necrosis, seemingly cytoplasmic inclusions answering the histological criteria for the Guarnieri body. Both the characters and the distribution of the vaccinal lesions leave little doubt as to their specific nature. The characters are obvious from the subsequent descriptions. The distribution is not in agreement with that of any known type of accessory streptococcic invader since the organs affected mostly are those generally shunned by the blood-born streptococcus. Furthermore, a check on the vaccine content of the organ by subinoculation and a comparison between the organ content of virus and the blood content in the same animal would apparently eliminate any doubts.

Liver: Liver lesions occur in diverse forms. In the earliest foci the walls of the liver sinusoids are damaged and an exudate of precipitated albumen and fibrin occupies the space between endothelium and liver parenchyma. One might be

led to infer from these early capillary lesions, which are promptly followed by the appearance of fibrin plugs within the vessels, that the subsequently appearing, abscess-like foci are of thrombotic origin. However, before capillary thromboses occur even in the earliest lesions, one finds one or more punctate liver cell necroses, and it is the punctate character of these necroses which forces one to eliminate thrombosis as a causative factor in their genesis. The lesion is primarily parenchymatous. These necrotic liver cells undergo cytoplasmic hyalinization; they stain deeply pink with eosin-methylene blue or Giemsa stains; their nuclei become pyknotic or gradually fade out, the chromatin being reduced to a fine dust-like deposit. Neither nuclear nor cytoplasmic swelling ("ballonartige") has been seen.

As the lesion enlarges the capillaries become plugged over a wider area and the thrombosis may even extend into larger vessels. It is particularly in this case that one may be led to regard the lesion as thrombotic; however analysis forces us to abandon this suggestion since in several instances it has been possible to trace these wide-spread areas of hyalin necrosis and to follow their formation as fusions of originally punctate single cell necroses such as those shown in Fig. 1. They occur in rabbits grossly free from evidences of coccidiosis or verminous cysts. One noteworthy feature is the very low grade reactive process; a few polymorphonuclear leukocytes may appear in the thrombosed sinusoids and may invade the necrotic liver cells, but the appearance is that of a paralyzed, insignificant type of reaction; the leukocytes present exhibit marked fragmentation of their nuclei which rapidly degenerate into nuclear dust. Fibroblastic proliferation is absent in the acute stage. Guarnieri bodies have never been seen.

Suprarenal: The lesions of the suprarenal are quite similar to those of the liver, save that the broad zones of hyaline necrosis were but once encountered. The early foci are either of the nature of sharp punctate single cell necroses or isolated hemorrhagic foci of varying size. (Fig. 2). The parenchymatous cells undergo rapid hyaline degeneration; a fibrin exudate is thrown out about the capillaries; fragmented polymorphonuclear leukocytes invade the necrotic cells. The reaction may become fairly intense, whereupon the appearance is that of an abscess with intense necrosis. (Fig. 3). In the early stages there is evidence of stimulation of the suprarenal parenchyma and several mitotic figures per oil immersion field may be encountered in pink-staining, damaged cells. (Fig. 5). Ballooning degeneration has not been seen and no Guarnieri bodies were to be found. These lesions are not due to secondary bacterial invaders; they appear in animals injected intravenously or intratesticularly or directly into the liver—in animals without skin lesions to serve as a source for secondary microbic invasion.

Ovary: The ovarian lesions are widely distributed throughout all anatomic divisions of the organ. They are to be found in the stroma, they involve the interstitial cells, theca interna and externa, and corona radiata. They are of the usual hemorrhagic, necrosing, degenerative type (Fig. 4) with much fibrin deposit, and may call forth a considerable polymorphonuclear and monocytic reaction. In the ovary alone of all the viscera have structures been encountered which

answer all the histological requirements of the Guarnieri bodies. These cytoplasmic inclusions are pictured in Figure 6. One must emphasize, however, the difficulty of really interpreting these structures; necrosis is so acute and so extensive, cell fragmentation so wide-spread, bits of broken-up acidophilic cell detritus are so readily washed from place to place during the mere technical handling of material, that appearances may become very deceptive. We feel therefore some doubt as to the nature of the structures which answer the morphological requirements of Guanieri bodies, especially in view of the fact that in no other viscus have they been certainly encountered.

Testis: Testicular lesions involve tubules, interstitial tissues, and in one instance the tunic was affected, with the production of an acute vaccinal periorchitis. These specific lesions in no way differ from those of the ovary or suprarenal. Specific cell inclusions could not be identified. In addition, in many animals in which no specific testicular pustules were found, spermatogenesis was nevertheless in abeyance, a certain amount of isolated punctate sex cell necrosis was observed and although we are unable to state that these lesions are specific, they nevertheless are undoubtedly related to the vaccinia or to the generalized intoxication accompanying the disease. In the summary showing percentage incidence of lesions in various organs, these minor testicular findings have been regarded as non-specific and are therefore not included. Slight sex cell necrosis has been encountered in controls.

Lungs: In the lung the vaccinal lesions may readily be confused with those of rabbit pneumonia. They differ from the common pneumonic lesion in several respects: they are more sharply focal; they are not primarily associated with bronchi but rather with vessels. They occur as a pronounced inflammatory, necrosing process in the vessel wall, with edema, thick perivascular deposition, invasion of the vessel wall by polymorphonuclear leukocytes, greatly fragmented and largely necrotic. From the vessel the lesion spreads to the neighboring alveoli but is apparently slow in giving rise to exudates within the bronchi. The nature of the process is shown in Figure 9. Occasional localized pleural and subpleural lesions of the same type exist. The typical lung lesions have been but rarely encountered. In one animal an almost tubercle-like, sharply focal necrosing lesion was found, a necrosis with practically no reaction on the part of inflammatory cells and with no involvement of the bronchi. Trachea and esophagus have been uniformly negative.

Spleen: The splenic lesions do not partake of the pustular character of the typical vaccinal foci encountered in the lungs, liver, adrenal, ovary, testis, nor the dermal pocks. The most striking thing about the spleens is the very marked lymphocytic exhaustion. The Malphigian bodies are scanty and small; the pulp lymphocytes are reduced in number; occasionally germinal centers are seen without any surrounding zone of small lymphocytes. There may be necrosis of splenic capillaries with thick perivascular fibrin deposits (Fig. 7). Isolated cell necrosis,—of lymphocytes, polymorphonuclear leukocytes, and large phagocytic cells may

be found. Hemorrhage is common. Where necrosis has been present the splenic lesions have been classified as vaccinal; in spleens where the only feature has been the pronounced lymphocyte exhaustion the lesion has been regarded as accessory only. Numerous sub-inoculations with spleen might be required before these doubtful points could be decided. Lymph nodes contain lesions similar to those of the spleen and have been similarly regarded. Fig. 8 indicates the extensive necroses which may be encountered in the lymph nodes.

Bone Marrow: We have studied but one bone marrow. This marrow showed an extensive necrosing vaccinal osteomyelitis with destruction of all marrow elements. Very little reaction was present in comparison with the intensity of the necrosis. The reacting polymorphonuclear leukocytes were greatly fragmented.

Kidney: No specific lesions have been encountered in the kidneys examined. Nevertheless acute degenerative changes in the convoluted tubules and collecting tubules are very common. These changes consist in swelling, albuminous degeneration, the presence of tubule casts, and in one instance a rather puzzling lesion, provisionally interpreted as an infarct, but nevertheless a peculiar one, for instead of a sharp zone of necrosis with surrounding reaction, the necrosis was poorly outlined and isolated necrotic tubules extended in fan-like processes far outward from the zone of major necrosis into the surrounding region of normal tubules. Leukocytic reaction was conspicuous by its absence. It is therefore possible that this lesion was specific.

Percentage Incidence of Visceral Lesions

In rabbits bearing enhanced skin lesions the percentage incidence of visceral lesions is summarized in Table I.

TABLE I

Specific Necrotic Lesions in Rabbits with enhanced skin lesions

Organ	Number Examined	Number with Specific Lesions	Percentage
Ovary	4	3	75.0
Suprarenal	11	8	72.7
Lymph Nodes (Vicinity of Lesion)	11	6	54.5
Testicle	13	7	53.8
Spleen	15	6	40.0
Lung	21	4	19.4
Bone Marrow	1	1	100.0
Kidney	15	1*	6.6
Brain	8	0	0.0

* Doubtful.

Accessory lesions in rabbits with enhanced skin lesions have been found in 10 cases in the spleen (66 percent), (lymphatic depletion), in 13 cases in the lung (60 percent), and in 9 cases in the kidney (60 percent). It must be added that both specific and accessory lesions were present in practically 100 percent of rabbits dying of vaccinal infection. The lowering of this percentage is due to the introduction of figures from rabbits purposely killed, some of them while in a good general condition.

The results in animals injected intravenously, intratesticularly or into the liver are summarized in Table II.

TABLE II

Specific Necrotic Lesions in Rabbits Injected into Liver, Testis, and Brain

Organ	Number Examined	Number with Specific Lesions	Percentage
Ovary	1	1	100.0
Liver	7	3	42.9
Testicle*	10	4	40.0
Suprarenal	8	2	25.0
Spleen	8	2†	25.0
Lung	9	0	0.0
Kidney	10	0	0.0
Brain	1	0	0.0

* In the case of intratesticular inoculation, the lesions of the opposite testicle only have been taken into consideration.

† One doubtful.

Accessory lesions in these rabbits have been found in 6 cases in the lung (66 percent), 6 cases in the kidney (60 percent), and 5 cases in the spleen (63 percent), (lymphocytic depletion). In two rabbits injected into the brain and dying 3 days later, very wide-spread accessory lesions were found in the kidney and also in the intestine.[2]

In a total of nine rabbits, of which 5 were injected intravenously and killed after 4, 6, 7 and 9 days, in two animals dying with enhanced skin lesions and in two others dying 6 days after intratesticular injection, the organs were tested for the presence of the virus by applying

[2] The lymph nodes were never examined as there was no gross indication of any lesion whatsoever.

the various organs to the scarified skin of normal rabbits. That virus could be recovered from internal organs is shown in Table III.

TABLE III

Recovery of the Virus

Organ	Number Examined	Number from which Virus Recovered	Percentage
Ovary	1	1	100.0
Kidney	9	5	55.6
Testicle	8	4	50.0
Suprarenal	9	4	44.5
Liver	9	4	44.5
Lung	9	3	33.3
Spleen	7	2	28.9
Blood	9	1*	12.2

* Very weak eruption.

The histological examination of the same fragment of the organ which was tested for its virus content was in general agreement with the result of the inoculation. Nevertheless, there were some discrepancies, for virus was recovered from lung and spleen in three cases, where no lesions, specific or accessory, were found, and in three other instances lesions in liver, kidney and suprarenal were found, whereas no virus was recovered. Either the inhibitory effect of the organ or the possible antibody content may explain the apparent discrepancy. In three of the rabbits of injected intravenously, the neurovirus was inoculated after mixing with 2.0 cc. of rabbit testicle extract prepared as described in a previous paper (1). A very careful study of the organs of these rabbits from both the histological and the virus content point of view did not show more infection than in animals injected with the virus alone. Testicle extract therefore does not appear to enhance the action of intravenously injected virus.

DISCUSSION

It would be quite apparent that any statements as to organ susceptibility to vaccine virus must take several factors into consideration. If we test for the virus content of an organ by applying the organ to

the scarified skin of a normal rabbit we obtain a certain result. However we may be in error since the enhancing or inhibiting action of various normal organ extracts is so marked that these activities may be of more importance in influencing the take in the normal animal than is the actual virus content of the tested organ. Again in all probability when testing an organ for virus content we are inoculating a mixture of virus plus antibody; the antibody content may not be the same in all organs; the rate of antibody formation is perhaps quite different. Were one to separate by cataphoresis methods the virus from the antibody in the various organs, then quite a different organ virus content might be ascertained. However that may be, the simple application of the suspected organ to the scarified skin of the normal rabbit may give one index of so-called organ susceptibility. Were it possible to show that the enhancing or inhibiting effects of different organs are paralleled by the multiplication or suppression of virus within the living cells of these organs then one might adopt quite a different standard of organ susceptibility. Finally, the presence of specific histological lesions in different organs would seem to offer still a third criterion for estimating specific organ affinities.

Our charts as well as the results of Douglas, Smith, and Price demonstrate that nowhere does vaccine virus exhibit any clear-cut, selective affinity for organ groups of similar embryological origin. Every organ reacts to vaccine virus in its own peculiar manner. An organ may appear insusceptible so far as the actual presence of specific histological lesions is concerned, and yet, extracts of it enhance the action of vaccine virus when mixed therewith *in vitro* and injected into the skin of a normal rabbit; another organ may manifest specific vaccinal lesions, yet, *in vitro*, a similar normal organ may inhibit the action of the virus; an organ may contain virus but exhibit no histological lesion or may present a specific histological lesion but prove negative on sub-inoculation. Of all organs studied the testis alone yields a high percentage of specific lesions, gives a high percentage of takes on sub-inoculation, and has a marked enhancing action when its extract is mixed with vaccine virus and inoculated into the skin of normal rabbits. It is by far the most active of the organs we have tested.

SUMMARY

Rabbits bearing very intensive skin lesions resulting from the intracutaneous injection of neurovirus plus testicular extract show typical histological alterations in the gonads, suprarenals, liver, spleen, lung, lymph nodes, and bone marrow.

Similar but less wide-spread alterations are found after intravenous injection of neurovirus.

Although testicle extract injected intracutaneously with neurovirus has a marked enhancing action upon the activity of the latter, the same mixture injected intravenously yields no sign of any such enhancement.

The significance of these observations as regards the question of the ectodermotropism of vaccine virus is discussed, and the doctrine of specific organ affinities is considered.

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EXPLANATION OF PLATES

PLATE 16

FIG. 1. Liver. Zenker; phloxine-methylene blue. Focal hyaline liver cell degeneration. × 200.

FIG. 2. Suprarenal. Similar fixation, staining and magnification. Specific necrosing, hemorrhagic lesion.

FIG. 3. Suprarenal. Similar fixation and staining. × 53. Multiple specific, abscess-like, vaccinal lesions.

PLATE 17

FIG. 4. Ovary. Zenker; Giemsa. Hemorrhagic, necrosing lesion involving Graafian follicle and theca. × 110.

FIG. 5. Suprarenal. Zenker; Giemsa. Early necrosing lesion showing four mitotic figures as evidence of stimulation. × 220.

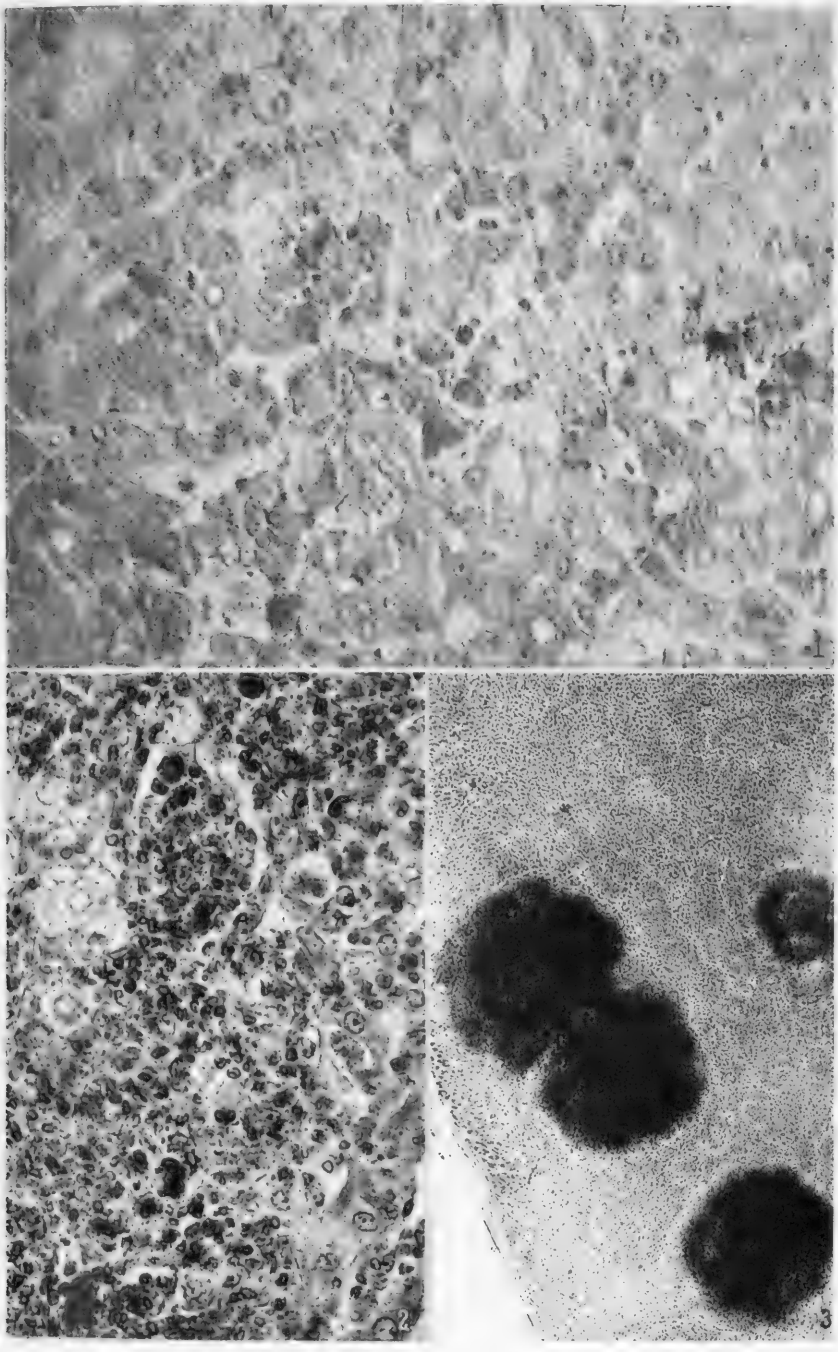
FIG. 6. Ovary. Zenker; Giemsa. Probable Guarnieri bodies. × 440.

PLATE 18

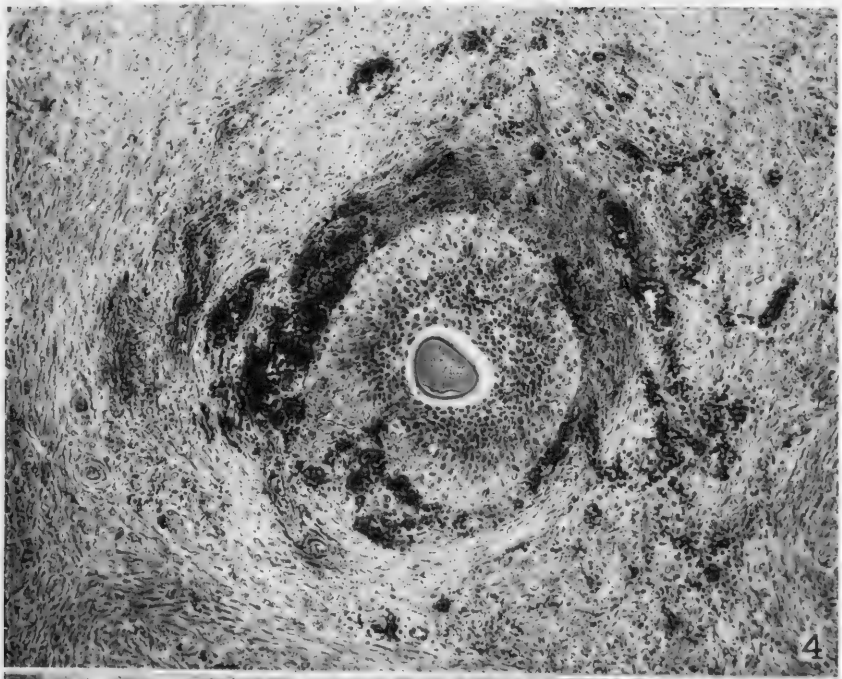
FIG. 7. Spleen. Zenker; phloxine-methylene blue. Diffuse fibrin deposit about vessels; marked diminution in lymphocytes. × 110.

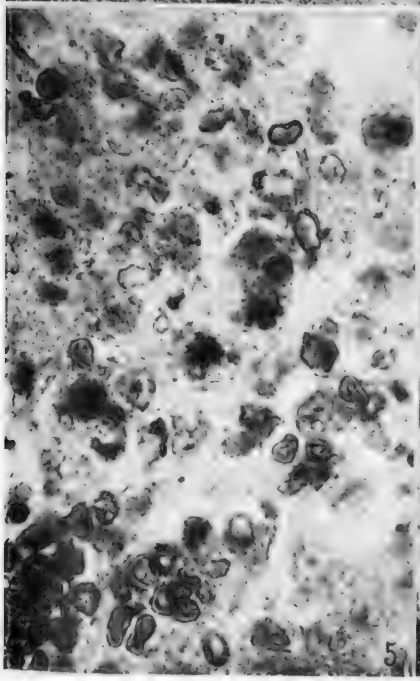
FIG. 8. Lymph node. Zenker; eosin-methylene blue. Diffuse necrosing lesion. Node taken from drainage area of an enhanced skin lesion. × 53.

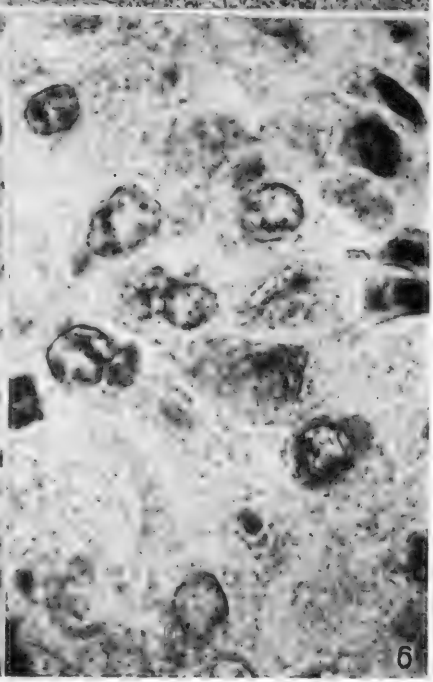
FIG. 9. Lung. Zenker; phloxine-methylene blue. Vascular and perivascular lesion interpreted as specific. × 200.



(Stewart and Duran-Reynals: Vaccine virus)







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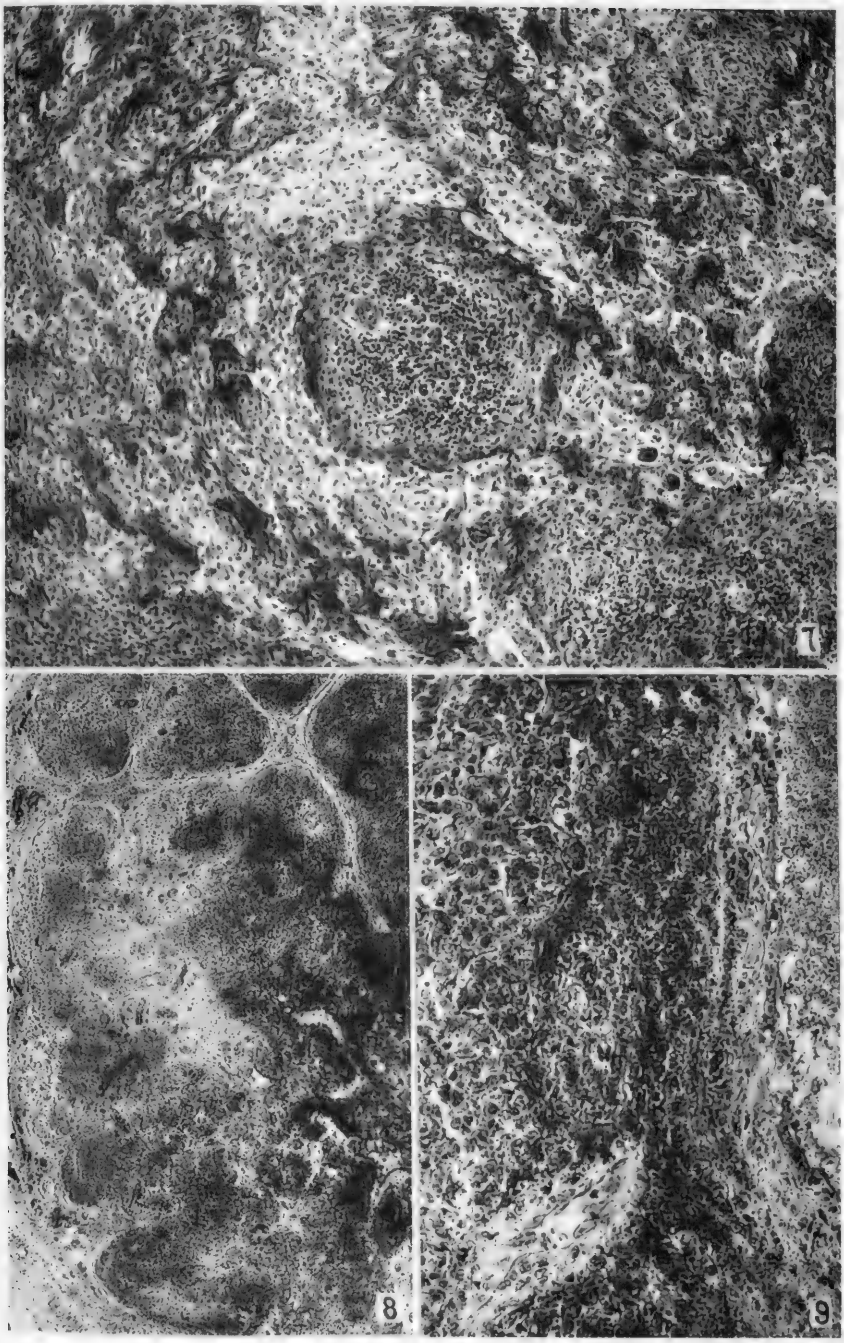

(Stewart and Duran-Reynals: Vaccine virus)

UNIV. OF ILL.

ETIOLOGY OF OROYA FEVER

XV. Effect of Immune Serum on the Course of Bartonella bacilliformis Infection in Macacus rhesus

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Plates 19 and 20

(Received for publication, June 14, 1929)

Very early in the course of the work on Carrion's disease, experiments were begun (Noguchi) to test the effect of an immune serum, prepared in rabbits, or obtained from recovered monkeys, on the course of experimental infection with *Bartonella bacilliformis* in *Macacus rhesus*. While these early results were not altogether promising, owing probably to the use of small doses of immune serum, there was some indication that the introduction of the serum simultaneously with the infective material inhibited the development of the infection, at least, temporarily (Table 1), the controls showing skin lesions and positive blood culture earlier than the treated animals.

Recently, studies were undertaken to determine the effects of larger doses (20 cc.) of convalescent serum given 24 hours before intradermal and intravenous inoculation of a highly virulent strain of *Bartonella bacilliformis* isolated from phlebotomi.[1] This procedure was found to have a marked inhibitory effect (Table 2). While the control animal developed severe skin lesions within 2 weeks, and its blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10,000 after periods of 10 and 17 days, the treated animals remained free from lesions for 25 days, and cultures of the blood were sterile. However, in two of the three treated animals, typical nodules eventually developed (after 25 days) at one or more sites of intradermal inoculation, and after 26 days the blood of one animal yielded cultures in a titre of 1:100. The third treated animal escaped infection.

[1] Noguchi, H., Shannon, R. C., Tilden, E. B., and Tyler, J. R., *J. Exper. Med.*, 1929, **49**, 993.

TABLE 1

M. rhesus No.	Date of inoculation 1926	Material inoculated	Mode of inoculation	Result		Treatment Date	Treatment Material	Result	
				Local lesions	Blood culture			Local lesions	Blood culture
59	Oct. 14	0.5 cc. susp. nod. Rh. 54 + 1.5 cc. immune rabbit serum. 2 cc. serum intrav.	Intradermal. Scarification.	No reaction until 20 days	− 5 days + 1:100,000 20 days	Nov. 26	3 cc. immune rabbit serum	Regression began Dec. 2	− Nov. 26 − Dec. 4
60	" "	Same except only 1 cc. serum intrav.	Same	Sl. transient reaction at one site after 20 days	− 5 days + 1:1,000 20 days				
61	" "	0.5 cc. susp. nod. Rh. 54 + 1.5 cc. convalescent serum Rh. 18 2 cc. serum intrav.	Same	No reaction	− 5 days Died of secondary infection Oct. 31.				
3-T	" "	Same as 61, but 1.5 cc. serum intrav.	Same	All inoculations positive in 12 days, mature nodules 20 days	+ 1:100,000 5 days + 1:100,000 20 days				

57 Control	Oct. 14	Mixture 0.5 cc. nodule susp. Rh. 54 and 1.5 cc. *normal rabbit serum*	Same	Definite 9 days	+ 1:100 5 days — 43 days	Nov. 26 1.5 cc. immune rabbit serum and 1 cc. culture subc.	No change Nodules still present Dec. 13 — Dec. 4
2-T Control	" "	Same	Same	Definite 5 days	+ 1:100,000 5 days	See Etiology of Oroya Fever. XIII.	
1-T Control	" "	Nodule susp. Rh. 54	Same	Definite 8 days Well devel. 22 days	+ 1:1,000 5 days — 43 days	See Etiology of Oroya Fever. XIII.	
55 Control	" "	Same	Same	Definite 8 days. Well developed 13 days	— 5 days — 43 days		
	1927						
83	Feb. 8	Susp. nod. tissue *M. rhesus* 78 Cultures P-5 strain 5 cc. immune serum intrav.	Intradermal, eyebrows and abdomen	No reaction	— 9 days — 23 "	Reinoculated Mar. 16, 1927, with suspension nodular tissue of *M. rhesus* 3A. No reaction.	
84A	" "	Same	Same	" "	— 9 days — 23 "	Killed because of tuberculosis Mar. 12, 1927.	
82	" "	Control, no serum	Same	" "	— 9 days — 23 days	" " " " " " "	
76	" "	Control, no serum	Same	Definite nodules 21 days. Reached 0.5 cm. in diameter	— 9 days + 1:100 23 days		

TABLE 2

Effect of One Intravenous Injection of Convalescent Serum 24 hrs. Prior to Inoculation

M. rhesus No	Pooled convalescent serum cc.	Date of inoculation 1928	Material inoculated	Mode of inoculation	Result	
					Local lesions	Blood culture
I-20	20	Nov. 10	Suspension nodular tissue from *M. rhesus* I-9 (Phleb. Str. 1)	Intradermal, 4 sites on abdomen. Scarification, 1 site Intrav. 1 cc.	One intradermal inoculation definitely positive after 25 days. Scarified area showed sl. reaction. Other 3 sites neg.	− 10 days − 17 " − 26 "
I-21	20	" "	Same	Same	All inoculations negative	− 10 days − 17 " + 1:100 26 days − 33 days
I-30	20	" "	Same	Same	2 intradermal positive after 25 days, 8 mm. in diameter.	− 10 days − 17 " − 26 " − 33 "
I-31	Control, no serum	" "	Same	Same	All intradermal inoculations positive in 14 days, reaching full development in 25 days. Severe reaction on scarified area. Died Dec. 10 (30 days)	+ 1:10,000 10 days + 1:10,000 17 "

Apparently the microorganisms had, in the two instances, remained dormant at the sites where they were intradermally introduced until the lapse of the period of passive immunity. This conclusion has been confirmed, by another experiment, as likewise the view that blood invasion was inhibited.

Four animals received an injection 24 hours before inoculation of 20 cc. of convalescent serum, another injection of the same amount 11 days after inoculation, and two subsequent injections of 4 cc. and 5 cc., respectively, 15 days and 26 days after inoculation. The control animals developed definite nodules in 11 days, which progressed rapidly and had reached very large size 12 days later. At this time the treated animals were still free from any lesions, and the blood was sterile. These conditions were maintained for a period of 38 days after inoculation (13 days after the last serum injection). At the end of this period, although blood cultures were still negative, definite nodules appeared at the sites of intradermal inoculation in 3 of the 4 monkeys; in the fourth animal nodules appeared 5 days later. The development of the lesions thereafter followed the usual course of experimental verruga, but *Bartonella bacilliformis* was recovered from the blood of the treated animals only in one animal (S-4) and then only once, 61 days after inoculation.

The experiment is recorded in detail in Table 3. It will be noted that the material used for inoculation was varied. Each monkey was injected intradermally at 3 or 4 sites on one side of the abdomen with a suspension of nodular tissue from a monkey infected with Phlebotomus Strain 1 of *Bartonella bacilliformis*, and each received similar inoculations on the other side with cultures of Phlebotomus Strain 3 or Strain 4. While the control animal for the nodule suspension responded in the usual way, only one of the 3 treated animals reacted to this material, though all showed the delayed reaction to the culture inoculations. But for the precaution taken to use varied material, the cultures in this instance proving to be of the maximum virulence, the results might have been interpreted as indicating that 3 of the 4 serum-treated animals were completely protected against infection. The one monkey which showed a delayed reaction (58 days) to the inoculation of the moderately virulent nodule suspension was evidently the most susceptible animal of the four, since it was the only one which was not completely protected against invasion of the blood by *Bartonella bacilliformis*.

The results recorded in Tables 2 and 3 indicate that while the

TABLE 3

Effect of Treatment with Convalescent Serum (First Dose Given 24 Hours Before Inoculation)

M. rhesus No.	Pooled convalescent serum cc. total	No. injections	Material and mode of inoculation	Local lesions	Blood culture
S-1	49	4	*Nodule susp. rhesus I-30* 3 sites intradermally	–	
			1 by scarification	–	– at 11, 19, 26, 46, 61 days after inoculation
			Cultures Phleb. Str. 1 3 sites intradermally	Very slight induration 39 days Mature in 66 " Recovery complete in 83 "	
			1 by scarification		
S-2	Same	Same	*Nodule susp. rhesus I-30* 3 sites intradermally	–	
			1 by scarification	–	– at 11, 19, 26, 46, 61 days after inoculation
			Cultures Phleb. Str. 3 3 sites intradermally	Definite nodules 39 days Mature in 51 " Recovery complete in 83 days	
			1 by scarification	+	
S-3	Same	Same	*Nodule susp. rhesus I-30* 3 sites intradermally	–	
			1 by scarification	–	– at 11, 19, 26, 46, 61 days after inoculation
			Cultures Phleb. Str. 4 3 sites intradermally	Very slight induration 39 days Mature in 66 " Recovery complete in 83 "	
			1 by scarification	–	

S-4	Same	Same	*Nodule susp. rhesus I-30* 3 sites intradermally	Nodule at 1 site 58 days Mature in 98 " 2.5 cm., pedunculated Still 8 mm. at 111 days	
			1 by scarification	−	− at 11, 19, 26, and 46 days, + at 61 days after inoculation
			Cultures Phleb. Str. 4 3 sites intradermally	Nodules at 2 of 3 sites in 46 days Mature in 83 " Recovery complete in 102 days	
			1 by scarification	−	
S-5 Control	None		*Nodule susp. rhesus I-30* 4 sites intradermally	Definite nodules in 11 days Mature in 23 " Recovery complete in 58 days	+ at 11, 19, and 26 days, − at 46 days after inoculation
			1 by scarification	++	
S-6 Control	None		*Cultures Phleb. Str. 3* 4 sites intradermally	Definite nodules in 11 days Mature in 23 " Recovery complete in 65 days	+ at 11, 19, and 26 days, − at 46 days after inoculation
			1 by scarification	++++	
S-7 Control	None		*Cultures Phleb. Str. 4* 4 sites intradermally	Definite nodules in 11 days Mature in 23 " Recovery complete in 58 days	− at 11 days + at 19 and 26 days − at 46 days after inoculation
			1 by scarification	++	

TABLE 4

Effect of Treatment with Convalescent Serum (First Dose Given 5 Days after Inoculation)

M. rhesus No.	Material and Mode of inoculation	Date 1929	Local lesions	Blood culture	Amounts of convalescent serum administered
S-8	Culture, Phlebotomus Strain 4.	Jan. 23	First definite nodule (4 mm.) 5 days after inoc. Nodules 10 mm., red, prominent, scarification +, 13 " " " Nodules average 15 mm., scarification + 19 " " " Nodules mature (10–20 mm.) 36 " " " Regression complete 67 " " "	+ 5 days − 13 " − 30 " − 43 "	20 cc., 5 days after inoc. 20 cc., 13 " " " 10 cc., 16 " " "
S-9	Same		First definite nodule (4 mm.) 5 " " " Nodules 8–9 mm. 13 " " " Same, scarification + 19 " " " Nodules mature (13–15 mm.) 36 " " " Regression complete 67 " " "	Same	Same

convalescent serum was not potent enough to protect completely against the inoculation of highly virulent cultures, it had a marked inhibitory action, as shown by the long delay in the development of the lesions, and that only occasionally did it fail to prevent invasion of the blood by the Bartonella.

The effect of large doses of convalescent *rhesus* serum given after local lesions had started to develop following the inoculation of cultures, was tested as follows:

In two *rhesus* monkeys, S-8 and S-9, treatment with convalescent serum was started 5 days after intradermal inoculation with a virulent strain of *Bartonella bacilliformis*, that is, when definite nodules had appeared, and when blood culture in both animals was positive in a dilution of 1:10. The serum was given intravenously in a dose of 20 cc. Notwithstanding the treatment, the nodules progressed rapidly, but 8 days later blood cultures were negative. At this time a second intravenous injection of 20 cc. of convalescent serum was given to each animal. Blood cultures continued negative throughout the remaining course of disease, but the local lesions progressed, and each animal was given another intravenous injection of 10 cc. of the immune serum on the 17th day. There was no perceptible effect of the serum treatment on the growth of the nodules, which matured about the 36th day after inoculation. Recovery was complete on the 67th day. At no test after serum was given were positive blood cultures obtained.

This experiment, recorded in detail in Table 4, shows that convalescent serum, given after the development of the nodules, sterilized the blood stream but had no effect on the nodule formation.

SUMMARY

Experiments are reported on the effect upon the course of experimental verruga peruana in *Macacus rhesus* of the injection of (1) small quantities of rabbit immune serum simultaneously with living cultures, (2) one large dose of convalescent monkey serum 24 hours prior to inoculation, (3) a similar preliminary dose followed by three subsequent injections of the serum, (4) three large doses of convalescent serum, following the inoculation. The convalescent serum was found (1) to prevent the multiplication of *Bartonella bacilliformis* in the blood in most instances, and (2) to delay the development of the skin lesions for considerable periods, when given before inoculation. When the serum treatment was not begun

until after the appearance of the skin lesions, it had no effect on the progress of the nodules, although the blood became free from *Bartonella bacilliformis*.

Since the severe effects of verruga peruana (Carrion's disease) are believed to be due to the multiplication of *Bartonella bacilliformis* within the blood, the injection of convalescent serum in cases of Carrion's disease in man would appear to offer promise.

EXPLANATION OF PLATES

PLATE 19

Figs. 1 and 2. Two of the control animals, S-7 and S-5, 24 days after inoculation.

Figs. 3 and 4. Treated animals, S-1 and S-2, 24 days after inoculation. Compare with Figs. 1 and 2.

Figs. 5 and 6. Treated animals, S-1 and S-2, 54 days after inoculation, when the lesions were comparable with those of the controls at 24 days (Figs. 1 and 2).

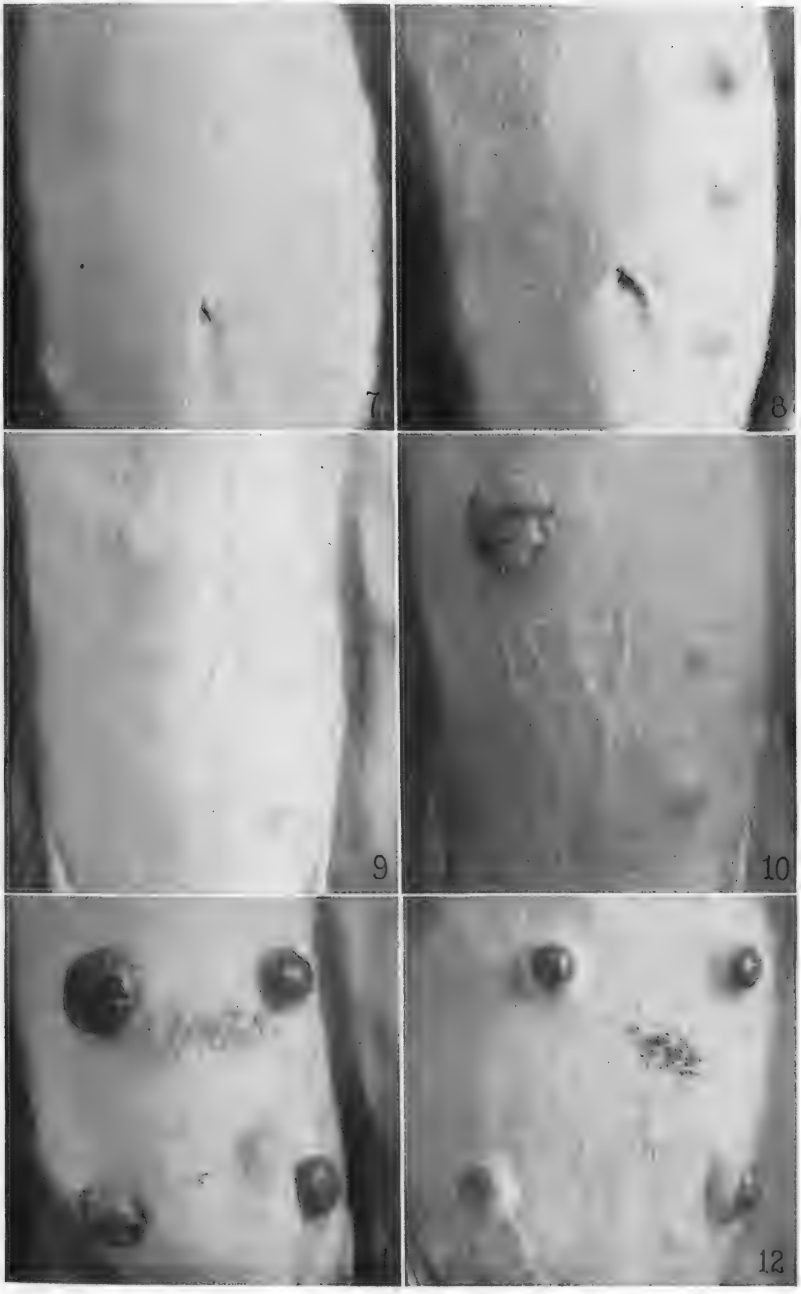
PLATE 20

Figs. 7 and 8. Treated animal, S-5, 24 and 54 days after inoculation, respectively.

Figs. 9 and 10. Treated animal S-4, 66 days and 100 days after inoculation, respectively. At 66 days the lesions had just become definite.

Figs. 11 and 12. Rhesus S-8 and S-9 (treatment begun after development of the lesions), 22 days after treatment was begun.

(Noguchi *et al.*: Etiology of Oroya fever. XV)



(Noguchi *et al.*: Etiology of Oroya fever. XV)

UNIV. OF MICH.

A PARALYTIC DISEASE OF GUINEA PIGS DUE TO THE TUBERCLE BACILLUS

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(Received for publication, May 23, 1929)

In a recent investigation in which a large number of tuberculous guinea pigs were under observation, a relatively high percentage of the animals became paralyzed early in the disease. These animals had each received subcutaneously 0.1 mg. of a culture of tubercle bacilli designated P. J. Out of 285 animals in the group between 30 and 40 manifested the paralysis. It had been noted previously among our tuberculous guinea pigs, but the cases had been so infrequent and scattered as not to attract serious consideration. In this particular experiment the disease complex made its appearance abruptly when the animals had been infected 2 months and, after new cases had developed for a period of about 10 days, it disappeared equally as abruptly. In considering the nature of the condition the most obvious possibility seemed to be that we were dealing with a tuberculous meningitis. The symptoms observed in our guinea pigs bore a close resemblance to those of experimental tuberculous meningitis of rabbits as described by Austrian (1), Kasahara (2) and Soper and Dworski (3) and of dogs as described by Manwaring (4). However, the occurrence of the malady in such a high proportion of animals, together with its abrupt onset and disappearance, made it seem essential to determine whether we were not dealing perhaps with some superimposed infectious disease of the central nervous system. Conceivably tubercle bacilli might be present and yet not be the essential cause of the paralytic condition. Because of this possibility it seemed advisable to maintain the paralytic disease for study by serial passage in guinea pigs. This was successfully accomplished by the intracerebral injection of emulsions of brain from animals showing the paralysis.

EXPERIMENTAL

There were three evident possibilities to consider in connection with the paralytic disease besides the tubercle bacillus. The first was infection with the filterable virus causing Roemer's (5) guinea pig paralysis; for the disease we were studying exhibited many points of similarity with that malady. A second possibility was that the animals had been infected with a low grade herpes virus at the time of inoculation, since the culture of tubercle bacilli used in this infection had been but quite recently isolated from human sputum. Third, the condition might have been an infection of the central nervous system by some visible organism other than the tubercle bacillus. The investigation was conducted with due reference to the points thus brought up.

The general plan followed in passing the paralytic condition from one animal to another was to remove the brain, under aseptic precautions, from a paralyzed guinea pig and after preparing an approximately 10 per cent emulsion of it in physiological salt solution to inject 0.1 cc. of the emulsion intracerebrally into a trephined normal animal.* The disease was maintained in this way through 9 successive groups of guinea pigs; and the clinical picture remained in all essential details the same throughout. The time elapsing between inoculation and the appearance of the first symptoms of illness varied from 8 to 27 days; and the tendency was for this period to shorten with each successive passage. The incubation period was remarkably constant for the animals of each passage group, however.

The disease as observed in guinea pigs infected experimentally was very uniform in its manifestations. For a period of 12 to 24 hours following the inoculation the animals appeared somewhat listless and disturbed, doubtless because of the procedure to which they had been submitted. Thereafter throughout the incubation period they remained bright, active and normal. The first symptoms noticed were a recurrence ot listlessness and some roughening of the fur. The animal sat quietly in its cage when not disturbed. There was usually some faulty coordination and the guinea pig had difficulty in getting to its feet when placed suddenly on the back. Very soon the incoordination became more marked. It was usually most noticeable in the hind quarters. Other symptoms referable to the central nervous system that were very frequently seen at this time were hyperesthesia, a tendency to move in circles, marked tremor, especially of the head, and a tendency to torticollis. A few animals showed a marked nystagmus. As a rule, the day following the onset of these symptoms a definite weakness of the hind quarters was evident, and there was often loss of sphincter control of both bladder and

* Ether anesthesia was practiced for all intracerebral inoculations.

rectum. This was followed very soon by a definite posterior paralysis, spastic at first and later flaccid. Very occasionally a paralysis of one or both fore legs preceded the posterior paralysis, and again, rarely, the condition resembled hemiplegia more than paraplegia. Following the early period of listlessness the animal appeared bright, and alert even when there was a complete posterior paralysis. One received the impression that the systemic reaction to the infection was very slight. Fig. 1 clearly illustrates the animals' state. As a rule, after 3 to 4 days of paralysis, its general condition suddenly became worse, it lost consciousness, the fur roughened, and, usually within 24 hours, death ensued. In 23 guinea pigs the disease was allowed to progress to a fatal termination and the average survival of these following the onset of the symptoms was 5.7 days, the extremes being 1 and 11 days. We had 6 animals die very suddenly, at the termination of

FIG. 1. Guinea Pig Y of the 7th serial passage showing complete posterior paralysis.

what corresponded to the incubation period in other animals of the same inoculation group, without having shown any symptoms whatsoever. No animal in our series that was inoculated with a fresh brain emulsion from either a "spontaneous" or experimental case was immune to infection. In animals living longer than 5 days after onset of the paralysis much loss of weight occurred. It took place, however, to some extent, in all of the animals, beginning late in the period of incubation. In a few, the onset of symptoms referable to the central nervous system was preceded by a very evident loss of weight. At no time was there any febrile reaction to the infection, although when the animal became moribund, the temperature occasionally became subnormal.

Pathology.—In animals in which the condition had been induced by the intracerebral injection of brain emulsion from a previous case a few macroscopic changes were consistently found at autopsy. On opening the calvarium an excess of cerebrospinal fluid was always encountered. This was usually slightly turbid. Nothing abnormal was evident in the brain itself, nor was there anything characteristic along the needle track. Rarely the meninges were adherent to the skull at the trephine region. They were usually somewhat thickened over the area of the mid-brain, pons and medulla at the base of the skull. Examination of the cord revealed no gross lesions.

Outside the central nervous system the only evidence of pathological alteration was found in the spleen. This was usually somewhat larger than normal and was studded with hyperplastic follicles.

Microscopic examination of paraffin sections of brain stained by the usual methods showed a great abundance of acid-fast bacilli in the thickened pia mater but none elsewhere. The condition was found to be a true meningitis with some extension into the encephalon along the lines of the larger blood vessels but elsewhere the encephalon was singularly free from infiltration or other lesions. The pia mater, especially at the base of the brain, was definitely, but not extremely, thickened. The exudate consisted of mononuclear cells, some of them lymphocytes, but large mononuclears chiefly. Necroses were lacking and giant cells and definite tubercle formation were not seen.

Bacteriology.—The brain emulsions as prepared for inoculation were examined for tubercle bacilli and other organisms. In the first of these emulsions to be studied tubercle bacilli were easily demonstrated by the Gabbet method, but in the material similarly prepared from later passages this was no longer possible. Many of the examinations were casual but some were prolonged and thorough.

Cultures of these brain emulsions were made on various media. No growth was obtained in any case on plain or blood agar. On Dorset's egg medium growth of typical tubercle bacilli was quite regularly obtained and these even from specimens in which no tubercle bacilli had been demonstrable in stained smear preparations. Cultures of brain emulsion in a modified Noguchi Leptospira medium (guinea pig serum was substituted for rabbit serum) showed an opalescent clouding of the upper portion of the medium after incubation at 37.5°C. for 10 days to 2 weeks. Later a granular sediment appeared. The upper growth was found on examination to be a pleomorphic Gram negative bacillus. It was non-acid fast by the ordinary Ziehl-Neelsen technique, but when decolorized after the method of Gabbet showed some acid-fast forms. The granular sediment was entirely non-acid-fast. Such cultures were recovered from nine different guinea pigs,—all that were so cultured. After 3 passages on media, guinea pigs were inoculated intracerebrally, some with the upper, diffuse growth and others with the sedimentary growth from one of these cultures. All developed the typical posterior paralysis with its accompanying symptoms. Animals inoculated subcutaneously with the upper portion of the culture developed within 4 days nodules the size of a

split pea at the site of inoculation. This was followed very soon by the appearance of large, shotty, inguinal lymph nodes. The nodules at the site of inoculation were discharging a cheesy pus within 2 weeks, and numerous organisms similar to those that had been grown in Noguchi media were found in this caseous material. Autopsy of these animals 2 months later revealed the presence of lesions typically encountered in guinea pig tuberculosis. The organism isolated and cultured in Noguchi medium was obviously a tubercle bacillus despite its atypical staining reactions.

Susceptibility of Rabbits.—Recourse was had to inoculations into rabbits in order to determine whether we were dealing with a mild form of herpes encephalitis, or with Roemer's virus which is described as specific for guinea pigs. Two rabbits were inoculated intracerebrally with brain emulsion from guinea pigs of the 3rd and 7th experimental serial passages. The animals developed a typical posterior paralysis with loss of bladder and rectal sphincter control, after incubation periods of 25 and 39 days respectively. There was nothing in their disease to suggest herpes encephalitis. Brain emulsions from these animals injected intracerebrally produced the disease in a second series of rabbits.

Non-Filtrability of the Infectious Agent.—Five Berkefeld N filtrates of brain emulsions from experimentally infected guinea pigs have failed to produce the disease when injected intracerebrally into 16 guinea pigs and 1 rabbit. Three of these filtrates were prepared by merely grinding the brain with sterile sand and salt solution in a mortar and then filtering; one was prepared by grinding in the same way, centrifuging the material, and passing it through cotton and paper before filtration; and in one, after the grinding, the preparation was shaken for half an hour with sand and glass beads and filtered first through cotton and paper and then through a Berkefeld N filter.

DISCUSSION AND SUMMARY

The experimental data collected during this study of a transmissible type of paralysis developing in tuberculous guinea pigs indicate the condition to be a true tuberculous meningitis. We have been able to rule out the possibility that it is due to a non-tuberculous infection of the central nervous system caused by Roemer's virus, or by an atypical herpes virus, or by some bacterium other than the tubercle bacillus. Roemer's virus and herpes could be eliminated from considera-

tion when Berkefeld N filtrates of infectious brain emulsions proved incapable of reproducing the disease. Furthermore, rabbits could be infected as they cannot with Roemer's virus, and the disease elicited in rabbits bears no semblance to herpes encephalitis. No organism other than the tubercle bacillus could be obtained on culturing brain or brain emulsions from experimental cases, and no others were seen in examining fresh smear preparations from the central nervous system. In a modified Noguchi medium a tubercle bacillus possessing atypical staining properties was obtained. This organism was capable of producing the typical paralytic disease when injected intracerebrally into guinea pigs, and also generalized tuberculosis in animals inoculated subcutaneously with it.

Typical tubercle bacilli were readily demonstrable in sections of the meninges from animals with the disease, and culture of pieces of brain on Dorset's egg medium usually yielded a growth of tubercle bacilli. Only in the first of the experimental passages, on the other hand, was it possible to demonstrate acid-fast organisms in fresh smear preparations from the central nervous system. This fact and the attributes of the atypically staining organisms encountered in the cultures in Noguchi media will be considered more fully in a subsequent publication.

In view of the much discussed question of the filtrability of the tubercle bacillus our observations concerning the failure of this organism to pass a Berkefeld N filter are of interest. No animal in our series inoculated intracerebrally with brain emulsion from either a "spontaneous" or experimental case of tuberculous meningitis failed to develop meningitis, and that rather acutely, while no animal in our series injected with a Berkefeld filtrate of brain emulsion has developed tuberculous meningitis or any other form of tuberculosis. In connection with this observation it must be recalled that the organism was atypical in respect to its staining qualities at least.

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THE CULTURAL AND STAINING REACTIONS OF A STRAIN OF THE TUBERCLE BACILLUS PRODUCING PARALYSIS IN GUINEA PIGS

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In a preceding paper by Shope and Lewis (1) account is given of a transmissible paralytic condition arising in tuberculous guinea pigs. It was believed possible that it had resulted from secondary infection with some neurotropic "virus." The methods of study adopted were designed primarily to establish or discredit this conception. The departures from the conventional technique so long rigidly adhered to by students of the tuberculosis problem have given rise to unexpected and significant results, one aspect of which it is the purpose of this paper to develop.

OBSERVATIONAL

The culture of the tubercle bacillus with which we are here concerned (Designated P. J.) was isolated in the summer of 1927 from the sputum of a colored adult male with chronic pulmonary tuberculosis, in the tuberculosis wards of the Philadelphia General Hospital, Philadelphia, Pa. The sputum showed many acid-fast bacilli when stained by Gabbet's method. Some of it about 24 hours old was injected subcutaneously into a number of guinea pigs, and these developed generalized tuberculosis in the typical manner. Four to six weeks after inoculation cultures were made on Dorset's egg medium from the spleens of several of these animals. No contaminations were encountered. The cultures developed rapidly and vigorously for the most part. After six weeks growth a representative tube was selected and transfers were made to Dorset's egg medium. After another eight weeks the second series of tubes were used for the inoculation of the large series of guinea pigs referred to in a preceding paper (1). The disease developed characteristically in them, except for the unusual occurrence of paralysis in some of the animals. The general virulence of the culture as estimated by the average length of life of the large series of animals was neither especially high nor low.

The culture P. J. has been maintained by rather infrequent transfers on Dorset's

egg medium and presents typical appearances thereon. The culture is acid-fast except that after decolorization by 30 per cent nitric acid followed by alcohol, counterstaining with Loeffler's methylene showed a few blue staining rods. It has not up to now been completely tested in all respects but there seems to be no reason to question the fact that the strain is a tubercle bacillus of human type.

When cases of paralysis appeared among the guinea pigs inoculated with the culture a successful effort was made to transfer the paralytic condition to other guinea pigs by direct intracerebral inoculation with brain emulsion from a case of the disease. This series of inoculations, from guinea pig to guinea pig by the intracerebral route, has been continued up to the present time. In the first emulsions made of brain and cord tubercle bacilli were easily demonstrated by the Gabbet method. But in the material similarly prepared from later passages this was no longer possible. Many of these examinations were casual but some were prolonged and thorough. Paraffin sections stained by the usual methods showed a great abundance of acid-fast bacilli in the thickened pia mater but none elsewhere.

Cultures of brain emulsions were made on various media and on Dorset's egg medium a typical growth of tubercle bacilli could usually be obtained. Those cultures of interest to the present discussion were made on a medium that is a variant of the one recommended by Noguchi for the cultivation of Leptospira. Ordinary nutrient dextrose agar was diluted 1 + 8 with distilled water, the diluted agar was boiled, cooled to 50°C., and 10 per cent of sterile guinea pig serum was added. Thorough mixture was effected at this temperature and the medium tubed in long narrow culture tubes (200 x 13 mm.). Portions of various of the emulsions of the nervous tissues were planted in these tubes, using a long capillary pipette in such a way as to form a thin streak from the bottom of the tube to the top along one side near the glass, with a small extra deposit on the upper surface of the semisolid medium. These plants were incubated at 37.5°C. in a moist thermostat for several months.

Certain of these tubes showed rapid growth of bacteria no two strains of which were alike and these were soon discarded. The others gradually showed the development of a faint cloud in the upper 10 mm. of the medium. This eventually became coarsely granular. It could be transferred to other tubes of the same medium and developed the same appearance. Exceptional tubes showed a faint haze toward the bottom with a clear intermediate zone.

When coverslip or slide preparations of the growth were fixed with methyl alcohol and stained with Loeffler's methylene blue, using heat to the point of steaming, and the staining process was continued for 3 to 5 minutes, rather definite, faintly staining, thin, small bacilli were found in increasing numbers as growth progressed. Stained by Gram's method in the usual way, employing Stirling's gentian violet, the bacilli were always Gram negative. The counterstains demonstrated them but faintly and it was frequently necessary to restain the preparation with Loeffler's methylene blue as above described to be sure of the presence of the

bacilli in the preparation. Such cultures were recovered from nine different guinea pigs,—all that were so cultured.

As stated in the preceding paper, the paralytic syndrome was readily reproduced by the intracerebral injection of these cultures in several instances. When culture material was introduced subcutaneously local lesions and lesions of the adjacent lymph nodes were produced, and autopsy of these animals two months later revealed the presence of lesions typical of guinea pig tuberculosis. This made it appear likely that the organism grown in Noguchi medium was a tubercle bacillus. Otherwise it was necessary to postulate the presence of a microörganism capable of inducing a disease simulating tuberculosis very closely. This question was now taken up directly.

When the cultures in the semisolid medium were restudied the following facts were made clear:

The bacilli however fixed and no matter how long and intensely stained with carbolfuchsin were always decolorized by 30 per cent nitric acid followed by alcohol, or by 3 per cent hydrochloric acid in 70 per cent alcohol; though it was indeed possible to make the staining so persistent that the background of culture material also retained a muddy red color after the application of acid, and then sometimes bacilli of the same dubious color could be made out with difficulty. The typical bright red of the Ziehl-Neelsen stain was never seen.

If the preparations were fixed with heat and stained by Gabbet's method a considerable number of bacilli retained the stain. If stained by Gabbet's method after fixation with methyl alcohol usually none of the bacilli retained the fuchsin stain but sometimes a few did. A few blue-staining bacilli were also usually seen. If the heat fixed or methyl-alcohol-fixed Gabbet stained preparations were further stained with Loeffler's methylene blue many blue stained bacilli were found. If the preparations were fixed in 4 per cent formaldehyde for 3 hours or more the number of bacilli retaining the fuchsin stain after Gabbet's methylene blue was often, but not always, greater than when fixation was by either heat or methyl alcohol.

Taking the matter from a slightly different standpoint: if the heat fixed preparations were stained with carbolfuchsin in the usual manner and decolorized by acid and alcohol separately it was found that 30 per cent nitric acid and 20 per cent sulfuric acid decolorized many but not all of the bacilli. The slightest application of alcohol, however, after these strengths of mineral acid removed all the stain. Even though much weaker mineral acids were used—5 per cent sulfuric, 3 per cent of either nitric or hydrochloric—alcohol later removed all the stain.

If Gram stain was employed according to the general plan of the Much method, with some modifications, the bacilli retained the stain. The essential feature of

this method is that the gentian violet is phenolized (2 per cent) and the stain when applied is heated. The iodin treatment is also prolonged and decolorization is done with equal parts of acetone and absolute alcohol. Under these conditions all of the bacilli retain the Gram stain. Every effort to show that some were Gram positive, others Ziehl-Neelsen positive has failed.

DISCUSSION

The preceding observations present two points of considerable interest with regard to the tuberculosis problem. The first relates to the identity of the culture and the criteria on the basis of which any microörganism is to be identified as a true tubercle bacillus. The second relates to the conditions of growth of the bacillus and their effect on its staining reactions.

The methods for identifying the tubercle bacillus as they now stand have been developed primarily in the interests of microscopic, clinical diagnosis. It has become a dogma in America (2) that if a bacterium will not, after staining with carbolfuchsin, withstand decolorization by 30 per cent nitric acid followed by alcohol it is not a tubercle bacillus. Heat fixation is always recommended for smear preparations but nothing is said as to the availability of other fixing methods. It is to be assumed however, since alcohol, formaldehyde, or chromium fixatives are more or less indiscriminately used for tissue work, that these are to be considered adequate for the demonstration of tubercle bacilli in smear preparations as well.

In Germany a similar dogmatic attitude is taken with reference to the critical value of 3 per cent hydrochloric acid in 70 per cent alcohol (acid alcohol) (3). To qualify as a tubercle bacillus a bacterium must resist decolorization by this agency. Our culture meets the classical requirements to the extent that it produces disease in animals of characteristic sort, that the bacilli in the fixed tissues resist decolorization, and the cultures on Dorset's egg medium present typical characteristics.

All this being true, it is astonishing to find that the smear preparations of the bacteria from the nervous system of the animals we have studied, when fixed in methyl alcohol, resist decolorization so little that their presence can pass unrecognized even though the weaker Gabbet method is employed for their demonstration, and that the culture in the semisolid medium yields organisms at wide variance

with the classical requirements. We are concerned with a bacterium which is moderately acid-fast, not alcohol-fast at all, and in which the particular method of fixation influences the result in ways not previously recognized. We are led by these observations to the conclusion that Much (4), and those who have followed him, are right in their contention that the tubercle bacillus may at times be positive to Gram stain and demonstrable by means of it, yet negative to the Ziehl-Neelsen method rigidly applied.* The weakness of Much's contention has in large measure been that the conditions under which such forms appear have never been accurately defined. Our observations when pushed further may supply this deficiency.

That the tubercle bacillus should be readily and easily grown in the semisolid medium of Noguchi is somewhat surprising, certainly interesting, and possibly useful. If other cultures, and cultures from other organs behave like the present one, the method would be the simplest and most certain way to recover the organism from the infected animal. Whether the virulence or other qualities of the microörganism are affected has yet to be determined. The medium contains no glycerine and hence differs from the other media recommended for the cultivation of the tubercle bacillus, with the exception of the coagulated egg of Dorset or its modifications from which the glycerine is sometimes omitted.

CONCLUSIONS

When a strain of human tuberculosis was carried from guinea pig to guinea pig by intracerebral inoculation the bacillus of tuberculosis could be readily recovered in the semisolid "Leptospira" medium of Noguchi simply modified by the substitution of guinea pig for rabbit serum. Whether even this modification is required remains to be determined.

The tubercle bacillus in these cultures and possibly in the nervous tissue as well differed from the classical type in that it was less acid-fast and quite lacking in alcohol fastness. However, it retained the Gram

* These observations have no bearing whatever on the further contention of the same school that there is a non-bacillary or granular form of the tubercle bacillus. Thus far, the bacillary form only has appeared in our animals and cultures.

stain as modified by Much. Staining reactions, as this culture of the tubercle bacillus shows, are not absolute qualities but are variable with conditions, a consideration which it may be important to recognize. Efforts to define these conditions more precisely are likely to be productive.

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THE REMOVAL OF AGGLUTININ FROM SENSITIZED MOTILE BACTERIA

Second Paper. The Agglutinative Properties of Water Washings

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Observations on the removal of agglutinin from sensitized motile bacteria were presented and discussed in a preceding paper.[1] Extraction of the sensitized test organism (*Bacillus aertrycke*) in a 5 per cent sodium chloride solution at a temperature of 60°C. resulted in the removal of considerable flagellar agglutinin while the somatic agglutinin was practically unaffected. Brief mention was made of an anomalous reaction which occurred when distilled water was employed as the extracting or washing medium. The reaction is described in detail in the following experiments together with some additional observations leading to a possible explanation.

General Methods

A normal actively motile strain of *B. aertrycke* (guinea pig paratyphi, Type II) recently isolated from the spleen of a naturally infected guinea pig was used as the test organism. Cultivation was carried out on moist Blake bottles, inoculated with a sufficient amount of young broth culture to cover the surface of the agar and incubated at 37°C. for 18 hours. Cultivation on a dry medium lowers the floccular agglutinability of the bacteria. The growth was removed with normal saline, transferred to a graduated centrifuge tube, and sedimented for an hour. The supernatant was discarded and the bacteria, still in a packed state, were rinsed once with saline.

The serum was from a rabbit immunized with the above strain of *B. aertrycke* and it contained both flagellar and somatic agglutinins. For sensitization of the bacteria the following materials were used: 0.5 cc. of packed bacteria, 0.1 cc. of

[1] Nelson, John B., *J. Exp. Med.*, 1928, **48**, 825.

antiserum, 2.4 cc. of saline. This formula gives the minimum proportion of packed bacteria required for the nearly complete removal of flagellar agglutinin with the serum dilution employed. It was about twice the amount required in the preceding work[1] with a different strain of *B. aertrycke* and its homologous antiserum of approximately the same titer. The bacteria were resuspended and the mixture incubated at 37°C. for 5 hours followed by overnight refrigeration.

The mixture was now centrifuged for 1 hour at the same speed and the supernatant removed. The packed sensitized bacteria were suspended in 2.5 cc. of distilled water and again centrifuged for an hour. The washing process was continued 3 to 5 times with fresh water after each sedimentation.

The agglutinin content of the absorbed antiserum and of the washings was determined by macroscopic agglutination with 0.5 cc. of antigen and 0.5 cc. of fluid dilutions. As a control, unabsorbed antiserum diluted and incubated similarly to the absorbed serum was tested in the same way. Two antigens designated whole and heated were used. The former was a fresh saline suspension of *B. aertrycke* and contained both flagellar and somatic components. The latter was a saline suspension heated to 100°C. for 30 minutes, washed, and resuspended in saline. It contained only the somatic component. Both suspensions were standardized to 2.4 with the Gates apparatus. Incubation was carried out at 37°C. for 3 hours followed by overnight refrigeration.

Experiments

The results of a typical experiment with the use of distilled water as the medium for the removal of agglutinin are given in Table I. The agglutinin titer of the absorbed antiserum mixture and of the several water washings is compared with that of the unabsorbed antiserum. If we take into account the initial dilution of the unabsorbed and absorbed antiserums, the actual titers are 25 times the figure given.

The unabsorbed antiserum agglutinated the whole antigen in high dilution. The type of clump was mixed, though predominantly flocular in the lower dilutions, while in the higher dilutions it was purely floccular. It agglutinated the heated or deflagellated antigen in the lower dilutions only with a purely granular type of clump. While the limit of agglutination for the whole antigen was identical with that of the antiserum used in the preceding work,[1] the intensity of the reaction in the lower dilutions was less with the present antiserum. The limit of agglutination for the heated antigen was one dilution higher with the earlier antiserum, and the intensity of the reaction was likewise more marked. The absorbed antiserum gave a slight, floccular agglu-

tination in very low dilution with whole antigen but no reaction with heated antigenen the lowest dilution possible. The single absorption afforded an approximately 99 per cent removal of both flagellar and somatic agglutinins.

TABLE I

Agglutination of B. aertrycke by Unabsorbed Antiserum, Absorbed Antiserum and the Water Washings of a Sensitized Suspension

Test fluid	Antigen	Test fluid dilutions										
		2	4	8	16	32	64	128	256	512	1,024	2,048
Unabsorbed serum	W*	++++ M	++++	++++	++++	++++	+++	+++	++	++	+	−
	H	++++ G	+++	++	+	±	±	−	−	−	−	−
Absorbed serum	W	+ F	±	trace	−	−	−	−	−			
	H	−	−	−	−	−	−	−	−			
1st water washing	W	± F	trace	−	−	−	−	−	−			
	H	−	−	−	−	−	−	−	−			
2nd water washing	W	++ F	++	+	+	+	±	±	−			
	H	++ F	+	±	±	±	−	−	−			
3rd water washing	W	+ F	++	+	+	±	±	−	−			
	H	++ F	+	±	±	−	−	−	−			
4th water washing	W	++ F	+	+	±	±	−	−	−			
	H	+ F	±	±	±	−	−	−	−			

* W = whole bacteria, H = heated bacteria (100°C.), M = mixed agglutination, F = floccular agglutination, G = granular agglutination. The same abbreviations are used in all the subsequent tables.

With the first water washing there was a slight floccular agglutination in the presence of whole antigen only. The following washings reacted quite differently. With the second washing there was a marked increase in both the intensity and the titer limit of agglutination with the whole antigen. The type of clumping was floccular. In addition there was a reduced but perfectly definite agglutination

in the presence of heated antigen. The type of clump was likewise floccular, with this difference that the aggregates were more loosely formed and became less compact upon standing than the usual floccular clumps. The subsequent washings duplicated the reaction though on an increasingly lower level.

When the above reaction is viewed as it stands, it appears that the suspension of sensitized motile bacteria in a salt-free medium results in the removal of agglutinin which gives a floccular type of agglutination not only in the presence of bacteria with their flagella attached but also in the presence of deflagellated bacteria. The occurrence of a floccular agglutination in the presence of deflagellated bacteria is contrary to the many observations which have been made on the agglutinative behavior of motile organisms.

Repetition of the above experiment with other strains of the same bacterial species and with another species of Salmonella (*B. paratyphi*, Type I), likewise of guinea pig origin, gave essentially similar results. The agglutinin titer of the second water washing fluctuated between 1:64 and 1:256 with a corresponding fluctuation in the titer of the other washings. In some cases the second and third washings were of equal titer. It may be noted that the intensity of the agglutination reaction with the water washings was regularly less marked than that of the extraction fluids in the preceding work.[1]

Upon consideration of the reaction it seemed possible that the flocculation which occurred in the presence of heated or deflagellated antigen might be due to the precipitation of material present in the washings. These, with the exception of the first, regularly showed a distinct turbidity which swirled visibly upon agitation. It was not due to bacteria, although the washings was never entirely free of bacteria. There was no sedimentation upon standing. The degree of turbidity tended to decrease with successive washings but a gradation was not always apparent. The stability of the washings in the presence of salt was determined by serial dilution with saline.

Sensitized bacteria were washed four times with distilled water and a portion of each washing heated to 70°C. for 30 minutes. The unheated and heated washings, in a total volume of 1 cc., were diluted serially with saline and incubated without the addition of antigen. The findings with the antigen-free washings are given in Table II.

There was no reaction with the first unheated washing. The others showed a graded flocculation with the settling out of a loose fluffy sediment which was easily dispersed upon agitation. There was never any flocculation with the heated washings. In appearance and amount the sediment was identical with that of the washings in the presence of heated antigen and in appearance with that of a pure flagellar suspension in the presence of specific antiserum. The material present in the washings likewise resembled flagella in its thermolability. Centrifuged suspensions of flagella, however, unlike the water wash-

TABLE II

The Reaction of Unheated and Heated Water Washings of a Sensitized Suspension upon Dilution with Normal Saline

Test fluid	State	Test fluid dilutions					
		2	4	8	16	32	64
1st water washing	Unheated	−	−	−	−	−	−
	Heated	−	−	−	−	−	−
2nd water washing	Unheated	++ F	+	±	±	±	−
	Heated	−	−	−	−	−	−
3rd water washing	Unheated	++ F	+	±	±	±	−
	Heated	−	−	−	−	−	−
4th water washing	Unheated	+ F	±	±	±	−	−
	Heated	−	−	−	−	−	−

ings, are nearly water clear. The presence of salt-precipitable material in all the washings save the first was definitely indicated.

The water washings always gave a more marked agglutination, both as to titer and to intensity, in the presence of whole antigen than in the presence of heated antigen. The reaction with the whole antigen was regarded as essentially a true agglutination due to flagellar agglutinin removed from the sensitized bacteria by the washing process. Since flagellar agglutinin is heat-stable at 70°C., at which temperature the washings no longer flocculate with saline alone, it was possible to test this assumption by heat inactivation.

Two series of 3 water washings from sensitized bacteria were employed. One series was heated at 70°C. for 30 minutes. The other was used unheated. Both were tested as usual against whole and heated antigen. The reactions with the heated washings are given in Table III. The unheated washings behaved essentially as before (Table I) and the results are not included.

The heated washings agglutinated the whole bacteria with the usual floccular clumping which was more compact than that of the typical clumping with pure flagella. As to titer, there was little difference

TABLE III

The Agglutination of B. aertrycke by the Heated Water Washings of a Sensitized Suspension

Test fluid	Antigen	Test fluid dilutions							
		4	8	16	32	64	128	256	512
1st water washing	W	++ F	±	−	−	−	−	−	−
	H	−	−	−	−	−	−	−	−
2nd water washing	W	++ F	++	++	+	+	trace	−	−
	H	−	−	−	−	−	−	−	−
3rd water washing	W	++ F	++	++	+	+	trace	−	−
	H	−	−	−	−	−	−	−	−

between the unheated and heated washings. Unlike the unheated washings, however, the heated ones failed to react in the presence of deflagellated antigen. It seems evident that the floccular reaction with whole antigen was a true agglutination due to the presence of flagellar agglutinin in the washings. That the water washings from the sensitized bacteria, with the exception of the first, contained flagella in suspension was a suggestive fact. The actual presence of flagella, under the conditions of the experiment, was difficult to demonstrate. Some observations, however, on the behavior of unsensitized motile bacteria are presented as contributing evidence.

0.5 cc. amounts of *B. aertrycke*, in centrifuge tubes, were resuspended with 2.5 cc. of distilled water and sedimented. Because of the difficulty in getting clear supernatants with unsensitized bacteria the time was increased to 90 minutes and

the speed from approximately 2,000 to 2,500 R.P.M. The bacteria were washed four times in this way. While the washings were not quite water-clear they failed to show the characteristic turbidity of the washings from sensitized bacteria. One lot was diluted serially with saline in a total volume of 1.0 cc. and incubated without antigen for the usual length of time. The other lot was diluted serially

TABLE IV

The Reaction of Unheated Water Washings of Unsensitized Bacteria upon the Addition of Antiserum and of Normal Saline

Test fluid	Dilution fluid	Test fluid dilutions						
		2	4	8	16	32	64	128
1st water washing	Immune serum	++ F	+	+	±	±	±	−
	Saline	−	−	−	−	−	−	−
2nd water washing	Immune serum	++ F	+	±	±	±	−	−
	Saline	−	−	−	−	−	−	−
3rd water washing	Immune serum	+ F	+	±	±	−	−	−
	Saline	−	−	−	−	−	−	−
4th water washing	Immune serum	+ F	±	±	−	−	−	−
	Saline	−	−	−	−	−	−	−

TABLE V

The Agglutinability of Whole and Washed Suspensions of B. aertrycke

Antigen	Serum dilutions									
	4	8	16	32	64	128	256	512	1,024	2,048
Whole bacteria	++++ M	++++	++++	+++	+++	++	++	++	+	−
Washed bacteria	++++ M	++++	+++	++	+	±	trace	trace	−	−

with saline in a volume of 0.5 cc., and 0.5 cc. of a 1:400 dilution of homologous antiserum added to each lot. Incubation was carried out in the same way. The washed unsensitized bacteria were standardized in saline and their agglutinability compared with that of unwashed whole bacteria. The same antiserum, used throughout, was employed. The observations on unsensitized bacteria are presented in Tables IV and V.

The washings from the unsensitized bacteria failed to flocculate upon the addition of saline. When dilute antiserum was added, however, flocculation occurred and the type of clumping was identical with that of the water washings from sensitized bacteria in the presence of saline. It was a typical flagellar agglutination. Unlike the series from sensitized bacteria, however, the initial washing contained flocculable material. That the bacteria present in these washings were too few in number to influence the reaction was shown by the following experiment.

Sedimented whole bacteria were rinsed and resuspended in saline. The suspension contained only bacteria; there were no free flagella. The suspension was then diluted with saline to a barely visible turbidity. A 0.5 cc. portion of this suspension was carried through five serial dilutions and 0.5 cc. of a 1:400 dilution of antiserum added to each tube. There was no agglutination in any dilution after the usual incubation.

The agglutinability of the washed unsensitized bacteria was considerably less than that of unwashed bacteria. As shown in Table V, the titer was decreased and the intensity of the reaction outside the zone of granular agglutination was markedly less with the former. The above observations point to the removal of a certain number of flagella from the bacteria with each washing manipulation. Upon centrifuging, the bacteria are sedimented while the flagella remain free in the supernatant. With the addition of specific antiserum flagella and agglutinin unite and a floccular agglutination results.

DISCUSSION

The preceding observations have proved readily reproducible with the technic outlined. An interpretation linking them together and offering a possible explanation of the described anomaly is presented:—

The resuspension of sedimented motile bacteria in a fluid medium results in the mechanical removal of some of the attached flagella. If the bacteria are unsensitized the flagella remain in suspension after centrifugation, and the clear supernatant gives a typical flagellar agglutination upon the addition of specific antiserum. If the bacteria are sensitized the free flagella, which are in combination with agglutinin, clump in the presence of salt and are removed upon centrifugation. The initial water washing of sensitized motile bacteria contains suffi-

cient salt from the previous absorption mixture to cause clumping of the flagella. The clumped flagella are removed by sedimentation and consequently the supernatant is inactive, failing to flocculate upon the addition of saline. The subsequent water washings are salt-free and the freed flagella, either with attached or freed agglutinin, remain in suspension. The water washings of sensitized bacteria are cloudy at this stage owing possibly to minute aggregates of flagella which are too small for sedimentation.

That agglutinin in some form is removed from flagella in the salt-free medium seems apparent. Nothing definite can be said concerning its physical state other than the fact that it is again able to unite with whole bacteria, causing a clear-cut agglutination in moderate dilution.

The water washings of sensitized bacteria, at this stage, also flocculate upon the addition of saline alone. The flocculation is in reality a flagellar agglutination which was previously inhibited by the absence of salt. In the presence of salt, recombination of flagella and agglutinin, or union of flagella already in combination with agglutinin, occurs, with subsequent clumping.

It is maintained that the floccular reaction which results when the salt-free water washings are mixed with a saline suspension of heated bacteria is in reality a flagellar agglutination and is entirely independent of the added antigen. Flagella and flagellar agglutinin are both present in the salt-free washing fluids. Salt is supplied by the added bacterial suspension (heated) and flagellar agglutination results. In the presence of whole bacteria agglutinin may be diverted from the free flagella by the excess of those attached to the bacteria causing under such circumstances a bacterial agglutination.

As regards the differential removal of agglutinin it may be said that flagellar agglutinin in a state capable of again causing flocculation of whole bacteria may be freed from sensitized motile bacteria by washing with salt-free water. With the degree of sensitization brought about in the work here reported somatic agglutinin, on the other hand, is not demonstrable in the washings.

SUMMARY

The salt-free water washings of a sensitized motile bacterium (*B. aertrycke*) were found to cause a floccular agglutination in the

presence of both whole and deflagellated antigen. Evidence was presented that the water washings when salt-free contained flagella and flagellar agglutinin and that clumping occurred upon the addition of saline. The floccular reaction in the presence of deflagellated bacteria was regarded as the agglutination of flagella present in the washings. In the presence of whole bacteria, however, actual bacterial agglutination resulted.

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THE EFFECTS OF LOSS OF GASTRIC AND PANCREATIC SECRETIONS AND THE METHODS FOR RESTORATION OF NORMAL CONDITIONS IN THE BODY

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From careful experimental (1, 2, 3) and clinical (4) studies, it has become well recognized that continued loss of gastric juice may lead rapidly to severe or fatal dehydration and alkalosis, due to secretion into the stomach with subsequent loss of large amounts of water and chlorine ion, together with a considerably smaller, but still significant amount of fixed base. Theoretically the most effective method of relieving such changes should be the intravenous administration of adequate amounts of a solution of the same inorganic composition as gastric juice, i.e. a solution in water of HCl and NaCl in 100 mM and 30 mM concentrations respectively. Practically, however, administration of 145 mM or .85% NaCl (physiological salt solution) in sufficient quantity is quite effective. This is due largely to normal renal activity which permits excretion of excess of fixed base in the form of $BHCO_3$ during the period after the normal fixed base level has been restored and while the normal Cl concentration has not yet been reached. The general use of salt solution in such cases, therefore, is well justified from both the theoretical and practical viewpoint.

In certain conditions, however, which include loss of combined gastrointestinal secretions, as for instance intestinal obstruction or fistula, and severe diarrhea, dehydration may be accompanied by less marked alkalosis, or even by severe acidosis, due presumably to greater loss of fixed base than fixed acid in the unabsorbed digestive secretions, together with the accumulation of such acids as lactic, phosphoric, sulfuric and occasionally ketone, dependent upon inadequate circula-

tion, urinary excretion and carbohydrate metabolism. Theoretically the most effective method of relieving such changes should be parenteral administration of adequate amounts of water, base, bicarbonate and chloride (together with dextrose in the case of ketosis) in the proportions in which they exist in the gastrointestinal secretions lost. Difficulties arise, however, in applying such treatment because our knowledge as to the normal proportion of water, fixed base and fixed acid in the combined intestinal secretions is not as complete as in the case of gastric juice, and because of the difficulty of preparing sterile and non-irritating solutions containing sufficient amounts of all the substances lost. Practically, however, excellent relief of dehydration and acidosis has frequently been seen following the administration of large amounts of saline or Ringer's solution, with or without the addition of dextrose, the reason again being normal renal activity which permits, in this instance, excretion of the excess of fixed acid (Cl^-) in combination with manufactured base (NH_4^+) with resultant retention of fixed base (B^+) for combination with HCO_3. However, in many instances of severe diarrhea associated with persistent oliguria (5) as well as in cases of nephritis (6) Hartmann noted that the administration of large amounts of Ringer's solution was not followed by adequate restoration of $BHCO_3$. By following closely the chemical changes taking place in the blood plasma of such cases and administering Ringer's solution, glucose and sodium bicarbonate individually, as needed, he came to feel that the results obtained were distinctly superior to those obtained by the administration of Ringer's solution and dextrose alone.

The proper dosage of alkali given in this way, however, could only be ascertained with certainty by means of chemical examination of the blood, which was a distinct disadvantage in prolonged cases of diarrhea and acidosis in small infants. In addition, to be non-irritating enough to be given subcutaneously or intraperitoneally, sodium bicarbonate solution had to be sterilized by Berkefeld filtration and brought to approximately pH 7.4 by means of the addition of carbon dioxide. Such difficulties, it was felt, restricted considerably the use of alkali outside of hospital practice, even though its administration may have been unquestionably indicated.

With the realization that the normal kidney has a most marked

ability to select for retention or excretion those substances needed or not needed by the body, provided a sufficient amount of water is available for adequate urinary volume, we felt that a solution, to be effective against loss of either gastric or intestinal secretions, should contain: (1) water in relative abundance (i.e. should be a somewhat hypotonic solution); (2) a sufficient source of Cl' and B^+ to overcome the effects of the loss of gastric juice; (3) a sufficient excess of fixed base over fixed acid which, while being available quickly enough without kidney intervention to overcome severe acidosis, would still be released slowly enough for excretion into the urine as $BHCO_3$ in the presence of alkalosis; (4) fixed base in proper physiological ratio as regards Na, K and Ca; and (5) an antiketogenic substance.

These conditions it was thought would be fulfilled by a solution of the following composition:

NaCl	= 95.0 mM B^+
KCl	= 5.0 mM B^+
$CaCl_2$	= 2.5 mM B^+
Na lactate	= 25.0 mM B^+

Such a solution has a total osmolar concentration of about 240 mM as compared with the normal plasma concentration of 320. The Cl content approximates the normal plasma concentration, the cations are in the same proportions as they occur in normal plasma and sodium lactate besides being antiketogenic provides an excess of fixed base over fixed acid when the lactate ion is removed by synthesis or oxidation. From previous clinical (7) as well as experimental (8, 9) studies, it would be expected that base would be released more or less quantitatively from combination with the lactate ion during the period of 3 or 4 hours after entrance into the blood stream, which should provide sodium bicarbonate rapidly enough in case of need but also slowly enough for excretion in case of excess.

Our experimental work on dogs was done (1) to gather more data as to the composition of normal digestive juices, (2) to study more thoroughly the effects of loss of such secretions and (3) to test the efficiency of the theoretically ideal solution mentioned above (which will hereafter be referred to as "combined" solution) in restoring the altered plasma to normal under experimentally produced conditions

of dehydration with alkalosis or acidosis. This communication will be confined to a consideration of the gastric and pancreatic secretions.

Chemical Methods

In all instances blood was obtained under oil by puncture of the femoral artery. Chloride was determined by the method of Van Slyke (10); bicarbonate by the method of Van Slyke (11); pH by the method of Hastings and Sendroy (12); protein by the micro-kjeldahl method (13); inorganic phosphorus by the method of Briggs (14); sulphate by the method of Dennis (15); lactic acid by the method of Friedemann, Cotonio and Shaffer (16); total base by the method of Stadie and Ross (17); glucose by the method of Shaffer and Hartmann (18); NPN by the method of Folin and Wu (19); urea by the method of Van Slyke (20) and the freezing point by a thermo-couple and galvanometer as described by Darrow and Buckman (21). The osmolar concentrations were calculated as described by Darrow and Hartmann (22).

Loss of Gastric Juice

1. Chemical changes induced by loss of gastric juice through repeated lavage

Experiment No. 1. Dog #2

In order to effect loss of gastric juice as simply as possible, and to avoid entirely operative procedures on the digestive tract which might influence quantitatively or qualitatively normal gastric secretion, dog No. 2 was permitted to eat and drink and live normally with the exception that some of the food together with the secreted gastric juice was withdrawn by gastric lavage. At least one or two feedings per day were permitted to be retained to prevent significant starvation, and water was kept at all times in the cage to provide adequate water intake during intervals between lavage. Water intake and output were not measured, but frequent observations indicated that the animal both drank and urinated fully as much as the normal animal.

The chemical changes following lavage are shown in Table I and Chart I. As would be expected, loss of the chlorine ion with increase in the bicarbonate ion (and pH) occurred in a few days time. Later, as loss of gastric juice continued, anhydremia developed, as indicated both by increase in serum protein and reduction of serum water contents. This dehydration occurred despite an apparently normal intake of water. During the third week when the Cl reduction and anhydremia were maximal, there also occurred a considerable increase

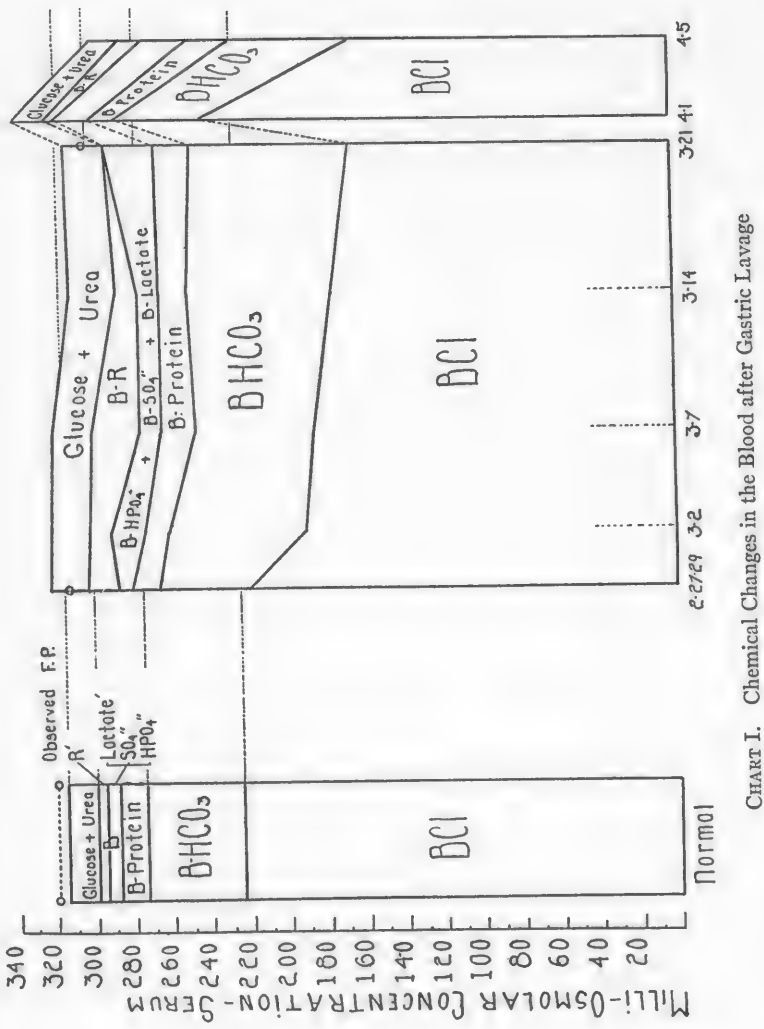

CHART I. Chemical Changes in the Blood after Gastric Lavage

TABLE I

Composition of Blood Serum of Dogs, as Affected by Loss of Gastric Juice and Therapeutic Measures

Dog No.	Date	Day of Drainage	Cl mM	HCO_3 mM	pH	Protein gms. %	Protein B^+ mM	$B\text{-}HPO_4$ mM	$B\text{-}SO_4$ mM	B-Lactate mM	Total Base mM	Undetermined Acid mM	Glucose mgs. %	N.P.N. mgs. %	Urea N mgs. %	H_2O content gms. %	Total Osmolar Concentration Observed mM	Total Osmolar Concentration Calculated mM	Remarks
Exp. 1. Loss of Gastric Juice by Lavage																			
2	2–27–29		103.8	22.1	7.40*	5.99	12.5	1.8	2.1	2.8	151	6	100*	43.3	33.8	94.2*	312	321	Lavage started
	3– 2–29		90.0	33.6	7.50*	5.63	12.6	3.4	2.1*	4.1	151*	5	90*	49.0	35.0*	94.5	—	319	
	3– 7–29		86.8	29.2	7.55	6.83	15.2	3.2	2.1*	1.5	150	12	90*	57.0	38.2	93.7	—	318	
	3–14–29		82.1	35.1	7.44	6.79	14.4	2.9	2.6	6.0	146	3	93	64.0	45.5	93.6		308	
	3–21–29		76.8	38.1	7.53	7.66	16.9	4.5	2.1	7.7	—	—	93*	67.0	39.2	92.9*	296	310	Lavage stopped
	4– 1–29		113.0	21.3	7.35	5.83	11.8	3.1	2.1*	5.4	159	2	67	41.3	28.4	93.6	300	334	" resumed
	4– 5–29		76.0	33.8	7.51	5.02	10.9	2.7	1.3	8.1	138	5	100*	46.0	26.1	92.8	274	295	
Exp. 2. Loss of Gastric Juice by Fistula—no Significant Treatment																			
28	2–12–29	1st	109.2	18.9	7.37	6.86	14.0	2.4	1.0*	4.2	144	−5	58	36.0	25.0*	94.1	—	310	
	2–16–29	5th	56.3	32.3	7.50*	6.48	14.1	8.7	3.0*	3.4	126	11	80*	214.0	175.0	92.7	—	316	Dog died 2–17–29

Exp. 3. Loss of Gastric Juice by Fistula—Administration of Glucose and "Combined" Solution

22	4–10–29	3rd	69.0	21.7	7.40*	7.59	15.8	4.1	2.0*	9.4	131	9	80*	70.0	55.5	93.4	298	282	
	4–11–29	4th	56.2	31.7	7.55*	8.58	19.0	6.8	2.0*	2.4	126	8	80*	127.0	89.0*	92.4	334	281	
	4–13–29	6th	99.0	21.4	7.40*	5.95	12.5	2.9	6.2	3.6	144	−2	93	27.0	17.4	94.5	303	297	
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1-38	5–31–29	3rd	76.7	35.0	7.55*	—	—	—	—	—	—	—	—	80.0	—	91.7			See text for account of symptoms and therapy
	6– 1–29	4th	81.2	31.5	7.50*	6.95	15.4	—	—	—	141	—	—	38.0	—	93.6			
	6– 3–29	6th	61.0	34.9	7.55*	7.87	17.6	—	—	—	137	—	—	93.0	—	92.6			
	8:45 a.m. 6– 4–29	7th	82.0	31.2	7.50*	5.73	12.4	—	—	3.1	136	—	—	33.0	—	93.9			
	2:00 p.m. 6– 4–29		88.0	29.5	7.45*	5.28	11.3	—	—	2.8	132	—	—	30.0	—	93.4			
	6– 5–29	8th	97.2	23.0	7.40*	4.58	9.6	1.8	—	—	133	—	115	16.0	—	94.3			

* Assumed values necessary for calculating total osmolar concentration and base-binding value of protein.

in blood lactic acid and a slight but appreciable increase in NPN, and decrease in total fixed base. Also during this period the osmotic pressure as determined by the freezing point diminished very slightly. The calculated total osmolar concentrations were in good agreement with the observed.

Ten days after gastric lavage was discontinued, the blood was again studied and found normal with the exception of BCl concentration, which was above the normal.

After four days of more intensive lavage during which time altogether eighteen stomach washings were made, the Cl ion concentration dropped to a level similar to that obtained after the previous three weeks of less intensive lavage,—namely to 76 mM. Bicarbonate ion increase was in part masked by increase of lactate. The total base was diminished 21 mM and the observed osmotic pressure dropped to a distinctly subnormal level. The calculated osmotic pressure was also subnormal but to a lesser degree. The NPN increased very little during this period. A slight fall of protein concentration occurred.

2. Effects of Loss of Gastric Juice by Fistula

Experiment 2. Dog #28

In this experiment we desired chiefly to note the rapidity and extent of plasma changes resulting from total loss of gastric juice.

On 2–12–29 the pylorus was resected under ether, the duodenal end closed and the stomach drained to the outside through a rubber tube. On 2–4–29, 3 mg. erganine were injected hypodermically to stimulate secretion of gastric juice for immediate collection and examination. 500 cc. of 5 per cent dextrose were given intraperitoneally on 2–15–29 and 2–16–29. Severe tetany and asthenia were present on the latter day. The dog was found dead on 2–17–29 the 6th day of drainage. From Table I it may be noted that by the 5th day of drainage plasma BCl concentration had fallen from 109 to 56 mM. Base bicarbonate increased only from 19 to 32 mM. This great discrepancy, 40 mM base, was well accounted for by the actual decrease in total base concentration plus the increase in phosphate and undetermined acid. NPN, 82 per cent of which was urea, increased from 36 to 214 mgs. per cent. The water content of the serum diminished without a corresponding increase in protein. The total calculated osmolar concentration changed only from 310 to 316 osm. mM. A sample of gastric juice obtained on the third day after injection of erganine showed the following composition; total Cl 133 mM, HCl 78 mM, total fixed base 27 mM, total N 507 mgs. per cent.

Experiment #3. Dog #22

In this experiment we proposed to investigate the effect on the prevention of dehydration of administration into the jejunum of: (1) dextrose solution and (2) dextrose solution and "combined" solution.

On 4–8–29 a gastric fistula was made under ether as in the previous experiment and in addition a jejunostomy was performed. Free drainage of gastric juice was noted on 4–9–29 and 200 cc. of 5 per cent dextrose were placed into the jejunum. On 4–10–29, 550 cc. were given. Tetany with convulsions developed on this day and blood was taken for chemical examination immediately after one of the latter. Changes similar to but not quite as marked as those noted in the previous experiment on the 5th day of drainage were found, with the exception that base bicarbonate increase was entirely absent, being masked by a very marked increase in lactic acid, presumably the result of convulsions. On the next day, three days after operation, BCl had diminished to 56 mM. Base bicarbonate had increased to 32 mM. Protein, phosphate, undetermined acid and NPN had increased significantly and total base had diminished to 126 mM. The total osmolar concentration observed was 334 mM, compared to a calculated osmolar concentration of 281. Signs of tetany were present on this day. In all respects, therefore, this dog appeared similar to the previous one on the day before death. On this day, however, 500 cc. of "combined" solution with 5 per cent added dextrose were given; on the next day 750 cc. were given in three divided doses, and on 4–13–29 the 5th day after operation, blood was examined chemically and found normal in all respects. At this time the dog seemed in very good shape, was able to walk and there was no signs of tetany.

Experiment No. 4. Dog #1–38

In this experiment we desired to confirm the results of the previous experiment.

On 5–29–29 a gastric fistula was made under ether by dividing the pylorus, closing the duodenal end and sewing the stomach end into the incision. Blood was examined two days later and the expected changes were found. On this day (5–31–29) 500 cc. of "combined" solution were given intraperitoneally. On the next day, the animal was more lively and the blood composition had changed in the direction of normal. No treatment was given on this day and on 6–2–29 in spite of the intraperitoneal administration of 500 cc. of 5 per cent dextrose, the dog appeared dehydrated and moribund. A relapse from the chemical standpoint was also noted (see Table I) despite the administration of dextrose solution. On 6–3–29, 500 cc. of "combined" solution were given intravenously followed by almost immediate symptomatic improvement. Later in the day 500 cc. were given intraperitoneally. On 6–4–29, 500 cc. were given intravenously at 11 a.m.

and three hours later blood was taken for study. At the same time urine was obtained. The blood showed a definite rise in BCl with a significant decrease of $BHCO_3$ and a slight fall in lactic acid. The urine was dilute. Its Cl concentration was 416 mM, $BHCO_3$ concentration 53.2 mM, NH_4 8 mM, while the total nitrogen concentration was 280 mgs. per cent. At 4 p.m. on 6–4–29 500 cc. more of "combined" solution were given intraperitoneally. This dosage was repeated on 6–5–29 at 12 noon, and at 3 p.m. on the same day blood was examined and found practically normal. The animal was killed and autopsied on 6–7–29. On that day the dog was able to walk; there were no signs of tetany. No infection was present.

It would seem therefore from the data of these experiments that the dehydration and alkalosis resulting from the loss of gastric juice is adequately relieved by administration of "combined" solution.

Loss of Pancreatic Juice

In these experiments pancreatic juice was obtained by the method previously described by one of us (23). (R. E.)

Very recently Gamble and McIver (24) published data concerning the inorganic composition of pancreatic juice as obtained by an open fistula and discussed the dehydration and acidosis which followed such loss of pancreatic juice. As might have been predicted, they observed a favorable effect on the dehydration and acidosis by the administration of sodium chloride and sodium bicarbonate. At the time of their publication we had made similar observations on two dogs concerning the nature of pancreatic juice and the effects of its complete loss by the method of intubation of the main pancreatic duct after ligation of all accessory ducts. In commenting on the two methods, Gamble and McIver concluded that the open fistula method was the superior, because they felt that premature death occurred in the intubated animals because of concommitant vomiting provoked by the intubated catheter. It would seem, however, that in reality the earlier death noted in the intubated animals (5 to 8 days as compared with 35 days) resulted from the more rapid dehydration and more marked acidosis due to the more extensive loss of pancreatic juice. In the method we used it was possible to obtain the entire 24 hour output from the entire pancreas and under sterile conditions. This is in contrast to the inevitable infection as well as the licking and consequent partial restoration of pancreatic juice in dogs with open fistulae. Infection itself leads to fibrosis of the gland and marked diminution of pancreatic secretion, an observation noted by most workers using open pancreatic fistulae. Vomiting in the intubated animals occurred only when fluid was taken into the stomach and is a manifestation of the gastric irritability which is part of the picture. Most animals seemed to realize this and took very little fluid and did not vomit. In a few dogs the desire for food and water was so great that they drank in large amounts and usually vomited afterwards.

Composition of Pancreatic Juice

Pancreatic juice as collected early after intubation, is faintly opalescent, sterile and without odor. Its total base concentration is roughly that of normal plasma, 150–160 mM and consists chiefly of sodium. Calcium averages about 4.5 mgm. per cent. Of the anions, HCO_3' is the most concentrated, averaging about 105 mM. Cl' averages about 40 mM. Protein varies from .8 to 1.9% averaging 1.2%. The albumin/globulin ratio is a little less than 1:2. Inorganic phosphorus concentration is usually less than 1 mg. per cent. Lactic acid concentration is also less than in plasma, averaging less than 1 mM. Undetermined acid averages about 5 mM. Glucose is absent and urea concentration approximates that of plasma. The total osmolar concentration both as determined and calculated also approximates that of normal plasma. The amount of pancreatic juice secreted during the first 24 hours averages between 10 and 20 cc. per hour and increases to 15–30 cc. per hour for the next few days.

The Effects of Continued Complete Loss of Pancreatic Juice

During the first 2 or 3 days of drainage, the average animal appears normal. As described in a previous paper, (23) he then develops loss of appetite, shows evidence of dehydration, but frequently not of thirst, becomes weak, drowsy, hyperpneic and dies on about the eighth day. Vomiting tends to occur particularly if food is gavaged or if the animal persists in drinking or eating, but may be entirely absent.

Plasma chemical changes are uniformly characteristic. (Table III and Chart II.) If vomiting is absent there occurs a steady fall in $BHCO_3$ until its concentration reaches about 7–8 mM when death intervenes. BCl also diminishes but usually only to a slight extent as compared with the diminution seen following loss of gastric juice. Protein concentration increases steadily and shortly before death may be extremely high, 10–13 per cent. Phosphoric, lactic and sulphuric acid concentrations increase several fold. Total base diminishes often to the extent of 25–30 mM. Undetermined acid tends to increase, but frequently remains extremely small. NPN concentration may exceed 200 mgm. per cent. Glucose in some animals is found low (in one case it was practically absent) while in others its concentration varies from the normal to 200–270 mgm. per cent.

TABLE II

Composition of Pancreatic Juice Obtained by Intubation

Dog No.	Date	(1) Day of Drainage	(2) 24 hr. Volume cc.	(3) BCl mM	(4) $BHCO_3$ mM	(5) Protein gms. %	(6) Protein B+ mM	(7) B-HPO_4 mM	(8) B-Lactate mM	(9) Total Base mM	(10) Undetermined Acid mM	(11) N.P.N. mgs. %	(12) Urea N mgs. %	(13) Total Osmolar Concentration Observed mM	(14) Total Osmolar Concentration Calculated mM	Remarks
Uncomplicated Total Loss of Pancreatic Juice																
20	12–20–28	2nd	550	54.7	84.5	1.9	4.7	0.0	0.1	154	10	34.0	24.0*	319	307	Juice sterile
	12–24–28	6th	365	76.7	66.1	1.2	3.0	0.3	0.7	149	2	44.0	31.0*	313	311	" "
	12–26–28	8th	320	73.3	61.1	1.4	3.6	0.2	0.2	141	3	83.6	58.5*	304	309	" "
	12–27–28	9th	145	62.2	60.7	1.1	2.7	0.0	0.4	130	4	220.0	154.0*	332	331	" " Day of death
Effects of Ringer's Solution Administration																
23	12–29–28	2nd	270	47.0	84.5	1.2	—	—	—	139	—	32.0	—	—	—	Juice sterile
	12–30	3rd	390	36.1	103.5	1.3	—	—	—	147	—	18.4	—	—	—	" "
	12–31	4th	390	42.6	83.7	1.2	—	—	—	144	—	12.0	—	—	—	" " 500 cc. Ringer's Sol.
	1– 1–29	5th	365	57.2	73.2	1.2	—	—	—	141	—	19.0	—	—	—	" "
	1– 2	6th	440	61.1	77.1	1.5	—	—	—	148	—	18.0	—	—	—	" " 500 cc. Ringer's Sol.
	1– 3	7th	600	53.0	85.8	1.4	—	—	—	158	—	24.0	—	—	—	" " 500 " " "
	1– 4	8th	720	51.4	84.8	1.2	—	—	—	158	—	14.4	—	—	—	" " 500 " " "
	1– 5	9th	610	56.2	80.0	1.3	—	—	—	143	—	25.6	—	—	—	" " 500 " " "
	1– 6	10th	390	46.0	79.0	2.1	—	—	—	150	—	29.3	—	—	—	" " 500 " " "
	1– 7	11th	370	36.1	82.0	1.6	—	—	—	139	—	24.0	—	—	—	" " 500 " " "
	1– 8	12th	320	36.9	79.0	1.5	—	—	—	135	—	18.0	—	—	—	" " 500 " " "
	1– 9	13th	325	30.9	86.0	1.6	—	—	—	140	—	23.2	—	—	—	Juice infected 500 cc. Ringer's Sol.
	1–10	14th	200	36.9	83.0	1.9	—	—	—	133	—	36.0	—	—	—	" " 500 " " "
	1–11	15th	200	42.1	83.0	1.8	—	—	—	140	—	26.0	—	—	—	" " 500 " " "

* Assumed values necessary for calculating total osmolar concentration.

Effects of "Combined" Solution Administration

27	1–29–29	2nd	300	38.9	104.2	1.9	—	—	—	163	—	57.6	—	—	—	Juice sterile
	1–30	3rd	550	37.9	103.7	1.4	—	—	—	153	—	38.0	—	—	—	" "
	1–31	4th	560	43.4	98.2	1.9	—	—	—	147	—	44.0	—	—	—	" infected 800 cc "combined" Sol.
	2– 1	5th	390	39.0	91.4	2.4	—	—	—	144	—	50.0	—	—	—	" " 400 " " "
	2– 2	6th	560	30.9	95.5	1.4	—	—	—	137	—	44.0	—	—	—	" " 400 " " "
	2– 3	7th	415	37.9	85.7	1.4	—	—	—	142	—	68.0	—	—	—	" " 400 " " "
	2– 4	8th	275	37.9	78.6	1.4	—	—	—	132	—	92.0	—	—	—	" " 800 " " "
	2– 5	9th	305	35.1	83.1	1.6	—	—	—	131	—	80.0	—	—	—	" " 800 " " "
	2– 6	10th	160	42.2	71.8	1.6	—	—	—	128	—	204?	—	—	—	" " 800 " " "
	2– 7	11th	100	56.8	42.0	?	—	—	—	135	—	—	—	—	—	" " 800 " " "
	2– 8	12th	160	73.5	45.7	?	—	—	—	149	—	427?	—	—	—	" " Dog killed
1	2–27–29	2nd	260	39.0	107.0	1.0	—	—	—	160	—	32.0	—	—	—	Juice sterile
	2–28	3rd	435	45.0	102.2	1.1	—	—	—	158	—	23.0	—	—	—	" "
	3– 1	4th	430	61.0	79.0	1.1	—	—	—	147	—	18.4	—	—	—	Juice infected
	3– 2	5th	400	65.0	63.7	1.3	—	—	—	142	—	51.3	—	—	—	" " 1000 cc. "combined" Solution on 3–2
																1000 " " " " 3–3
	3– 4	7th	200	66.0	41.7	1.4	—	—	—	135	—	260.0	—	—	—	" " 500 " " " " 3–4
																1000 " " " 3–5
																750 " " " " 3–6

The water content of the serum diminishes usually from 94 to 91 gms. per 100 cc. The plasma volume as compared with the cell volume decreases enormously, however, sometimes being only one-fifth the total blood volume. The total osmolar concentration both as observed and calculated changes but little.

If marked vomiting occurs, $BHCO_3$ loss may be partly or entirely masked, while BCl diminution is intensified.

Early infection of the pancreatic juice and of the pancreas may also alter the course of events somewhat, as mentioned earlier. Usually infection results in diminution of pancreatic secretion, and delays fatal changes somewhat (see Table II). In Dog ✱25 glucose was practically absent from the blood on the 13th day (day of death) and an extremely high concentration of lactic acid was present. Apparently either glycolysis occurred in vivo or else developed extremely rapidly after removal of the blood.

Renal activity (Chart II) is such as to compensate for the plasma changes. The urine diminishes in volume, contains almost no bound HCO_3 and therefore becomes highly acid, becomes free of Cl and concentrated as regards HPO_4, ammonia and urea. Fixed base is much diminished. A heavy trace of albumin and numerous granular casts are usually present.

The pancreatic juice changes somewhat in composition as dehydration and acidosis increase. (Table II, Chart II.) It diminishes in volume and becomes considerably less rich in base bicarbonate, but remains very alkaline as compared to the plasma. BCl increases somewhat in concentration. Its total fixed base concentration steadily diminishes but usually not as rapidly or to the extent that plasma base diminishes. NPN and urea continue to approximate their plasma concentrations, probably because of simple filtration.

It would seem therefore that secretion of pancreatic juice, as in the case of gastric juice secretion, continues more or less unaltered despite the fact that its loss leads to the rapid death of the animal. Renal activity while very important in compensating for loss of water, fixed base and Cl, is insufficient to prevent death in the absence of sufficient mineral intake.

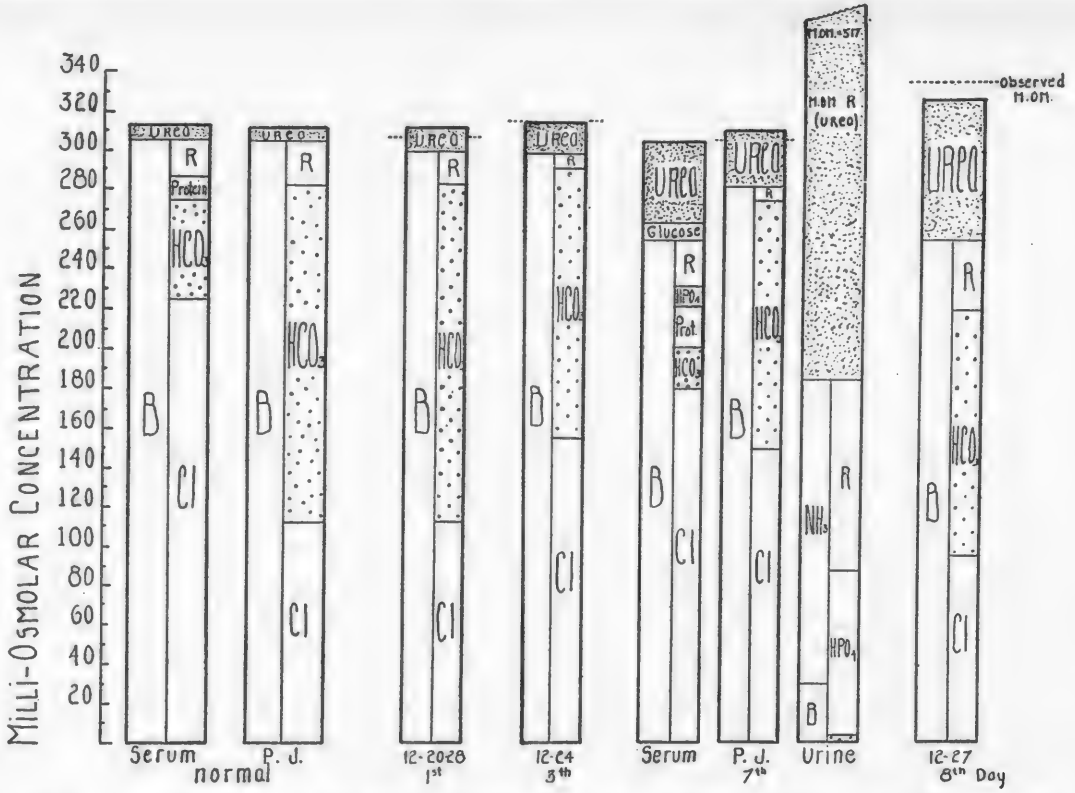

CHART II. Chemical Changes in the Blood, Pancreatic Juice, and Urine Associated with Continued Total Loss of Pancreatic Juice

TABLE III

Composition of Blood Serum as Affected by Complete Loss of Pancreatic Juice, Complicating Factors Such as Infection, Vomiting, and Incomplete Loss, and by Therapeutic Measures

Dog No.	Date	Day of Drainage	BCl mM	$BHCO_3$ mM	pH	Protein gms. %	Protein B+ mM	$B\text{-}HPO_4$ mM	$B\text{-}SO_4$ mM	B-Lactate mM	Total Base mM	Undetermined Acid mM	Glucose mgs. %	N.P.N. mgs. %	Urea N mgs. %	H_2O content gms. %	Total Osmolar Concentration Observed mM	Total Osmolar Concentration Calculated mM	Remarks
Uncomplicated Complete Loss of Pancreatic Juice																			
16	11–26–28	7th	98.8	6.2	7.17	8.49	15.8	4.1	2.0*	5.7	135	4	135	70.0	49.0*	92.2*	—	300	
20	12–26–28	8th	80.7	9.6	7.33	9.34	18.8	5.8	3.0*	3.0	117	−4	155	147.0	103.0*	90.4	—	285	
Complete Obstruction of Intubation Tube—Marked Vomiting—No Loss of Pancreatic Juice																			
19	12–15–28	4th	81.0	31.7	7.51	7.22	15.8	3.8	2.0*	4.8	173?	34?	148	68.2	47.8	93.2*	—	367?	
Early Infection of Pancreatic Juice																			
11	4– 6–29	14th	87.0	7.2	—	—	—	—	—	—	107	—	—	100.0					
25	4– 6–29	13th	97.8	14.0	7.30	5.50	10.8	3.9	1.9	16.5	158	13	20.0	70.0	49.0*	94.5	315	335	
3-10	4– 9–29	7th	80.0	7.7	7.15	10.8	19.9	5.5	8.6	6.6	122	−6	268.0	233.0	92.0	91.9	320	296	
7	5–21–29	5th	83.0	13.1	7.31	7.78	13.6	5.4	2.0*	1.5	133	14	80.0*	96.6	67.7*	91.6	—	306	
Effects of Administration of Ringer's Solution																			
23	12–31–28	4th	90.6	12.0	7.33	8.23	16.5	3.3	1.0*	5.0	140	12	146	31.6	16.0*	92.7	—	294	
	1– 5–29	9th	90.0	8.8	7.31	7.46	14.8	3.7	1.0*	3.0	129	8	127	46.7	24.0*	91.9	—	277	Therapy begun
	1–11–29	15th	95.8	14.2	7.32	6.56	13.1	2.7	1.0*	2.0	134	5	148	30.0	15.0*	95.0	—	278	

Effects of Administration of "Combined" Solution

27	1–28–29	1st	97.3	20.1	7.44	—	—	—	—	4.2	—	—	191	38.4	—	—	—	—	
	1–31–29	4th	86.4	9.4	7.30*	8.98	17.8	3.0*	1.0*	2.8	123	3	100*	63.0	44.0*	91.5	—	269	Therapy begun
	2– 8–29	12th	95.0	21.9	7.33	5.45	10.9	1.4	1.0*	2.2	144	11	97	27.0	13.5*	93.5	—	302	
1	2–27–29	2nd	103.2	19.6	7.35*	6.38	13.0	3.1	2.6	1.5	147	4	100*	30.0	15.3	93.9*	315	304	
	3– 2–29	5th	76.2	14.8	7.30*	8.12	16.1	4.9	3.0*	2.5	141	24	80*	123.6	69.0	92.5*	—	311	Therapy begun
	3– 7–29	10th	95.2	28.0	7.51	5.00	10.9	2.5	1.0*	1.5	148	9	100*	25.0	11.4	95.0*	—	304	

* Assumed values necessary for calculating total osmolar concentration and base-binding value of protein.

The Effects of the Administration of Dextrose, Ringer's and "Combined" Solutions

When only water and dextrose solution are administered, no noteworthy diminution of dehydration and acidosis or prolongation of life seems to occur.

Administration of Ringer's solution, however, (Table III) in amounts somewhat greater than the volume of the pancreatic juice lost has a decided beneficial effect. After administration of Ringer's solution to Dog #23 (Table II) was begun, pancreatic juice, which had diminished in volume and $BHCO_3$ concentration, again increased in volume and alkalinity. The dog showed symptomatic improvement. He became lively and his appetite returned. Urinary output increased and renal activity, largely through excretion of chloride bound to ammonia with resultant retention of fixed base, was sufficient to prevent plasma $BHCO_3$ from falling to a fatal level. It is to be noted, however, that $BHCO_3$ continued to diminish for a number of days after beginning the administration of Ringer's solution and then increased but did not nearly reach the normal level by the 15th day.

With the administration of "combined" solution almost immediate relief of dehydration and acidosis was accomplished (Table III). Kidney intervention, while necessary for the excretion of such accumulated acids as phosphoric and sulfuric and of excess non-protein nitrogen, was unnecessary for a rapid restoration to normal of plasma $BHCO_3$ and pH.

It would seem, therefore, that the "combined" solution is effective in relieving dehydration due to loss of fixed base and fixed acid, regardless of whether "acidosis" or "alkalosis" exists. Severe renal insufficiency should not prevent plasma $BHCO_3$ restoration. Theoretically, however, it might delay excretion of $BHCO_3$ excess.

An experimental study of this problem will be reserved for a later paper.*

SUMMARY AND CONCLUSIONS

The composition of gastric and pancreatic juices and the effects of their loss on the composition of the body fluids were studied. Loss

* A preliminary report on the clinical use of the "combined" solution was given by one of us (A. F. H.) at the Forty-first Annual Meeting of the American Pediatric Society.

of gastric juice by removing water and chloride ions only partly neutralized by fixed base results in dehydration and alkalosis.

Loss of pancreatic juice by removing water and a relative excess of fixed base results in dehydration and acidosis.

Normal conditions in the body may be restored after the loss of either gastric or pancreatic juice by the administration of a combined solution, which provides (1) water in abundance because of its hypotonicity (2) an adequate source of the fixed anion Cl' and of the cations Na^+, K^+, and Ca^{++} in proper physiological ratio and (3) an excess of fixed base over fixed acid in the form of B-lactate.

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